Biochemistry Of Grape Berries: Post-genomics Approaches To Uncover The Effects Of Water Deficits On Ripening

Rita Brito Francisco

Dissertation presented to obtain the Ph.D degree in Biochemistry, Plant Physiology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
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Knowledge Creation

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To my mother

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ABSTRACT

Grapevine (*Vitis vinifera L.*) is one of the most important fruit crops worldwide. In Europe, high-quality wine producing areas are traditionally non-irrigated. However, irrigation has become a wide-spread agronomical practice to overcome the deleterious effects of drought, high temperature and high evaporative demand that vines can be exposed to during the growing season. This is particularly true in the Mediterranean area, where the foreseen scenario predicts that water deficit may become a limiting factor in wine production and quality. Paradoxically, the imposition of mild to moderate water deficit (WD) via e.g. water deficit irrigation has been regarded as an agronomical tool that manipulates berry sensory characteristics, while maintaining yield.

Grape berries, which are described as non-climacteric fruits, undergo a complex biochemical suite of alterations during development and ripening that remain poorly understood, including the molecular events that control the onset of ripening. At harvest, grape berry quality is largely dependent on the sugar/acids balance within the berry flesh, and on phenolic compounds (e.g. flavonoids) in the grape skin, which contribute to wine colour, aroma and flavour.

The main aim of this thesis was to gain novel insights into the molecular mechanisms involved in grape berry ripening and of how the water status of grapevines may affect these processes. The study was conducted with berries collected in the field at different phenological stages using Aragonez, one of the most relevant grapevine varieties to viticulture in the Iberian Peninsula. The effect of grapevine water status on the regulation of some of the major metabolic alterations occurring in the grape berry exocarp during ripening was studied at the transcriptional and proteome levels. The impact of these regulatory changes on grape berry quality traits, namely anthocyanins and sugar contents were studied (Chapters II and III). Moreover, anthocyanins are some of the most important flavonoid compounds that accumulate during grape berry ripening. Therefore, a study of a putative *Vitis vinifera* vacuolar transporter of the ABCC subfamily of the ATP-binding cassette superfamily, VvABCC1, was also undertaken (Chapter IV).

Our results showed that the physiological impact of mild to moderate WD conditions, imposed by regulated-deficit irrigation (RDI) and even non-irrigation (NI) was not detrimental to the accumulation of sugars and anthocyanins, as compared to full-irrigation (FI) conditions. Nevertheless, WD hastened the accumulation of hexoses in both conditions (RDI and NI) whereas anthocyanins started to accumulate earlier under RDI conditions as compared to both NI and FI conditions. The examination of transcriptional data revealed few transcriptional changes which could potentially be associated with the early accumulation in RDI, as compared to NI berries. One such significant change was observed in *MYBA3*, a non-functional transcriptional factor that has been proposed to compete with the functional MYBA anthocyanin regulators. This gene was induced in the initial stages of NI conditions. The transcriptional data also suggested that WD had an impact on hormonal metabolism, namely ABA and ethylene, potentially involved in the regulation of ripening events. In addition, the data collected for the 'sugar signal` trehalose-6-phosphate suggested that it may be involved in grape berry maturation. Moreover, much of the findings presented at the transcriptional level provide clues for further functional analysis that could improve our understanding of grape maturation process.

At the proteome level, 74 proteins were identified, the majority of which could be functional classified as carbohydrate or stress-related proteins. The early accumulation of hexoses, which is marked under RDI conditions, is proposed to be important for the alterations observed at the proteome level. Eight proteins which were specifically up-regulated under RDI conditions during the onset of anthocyanins and hexose accumulation were assigned as putative 'signature' proteins that characterize the onset of Aragonez ripening. Although the bulk of the identified proteins have been described in previous *Vitis* studies, this work also provides new entries in the grape berry proteome catalogue.

A comparison of the transcriptome and proteome data strongly suggesting the involvement of post-translational regulation in ripening events, since some of the most significant alterations at the protein level, including a vacuolar invertase, a thaumatin-

like protein and a chitinase IV, were not corroborated at the transcriptome level. The biochemical characterization of VvABCC1, was demonstrated capable of transporting glucosylated anthocyanins and auxin conjugates into the vacuole *in vitro*, suggesting that ABC-transporters may be involved in grape berry ripening processes. Further considerations suggest that, within this role, VvABCC1 may be functionally compartmentalised: for the transport of anthocyanins into the vacuole in berry skin and as a vacuolar IAA-conjugate transporter in berry flesh.

These results provide a useful overview of the dynamic changes in the transcriptome and proteome of Aragonez grape skin during berry maturation, which suggests that both transcriptional and post-transcriptional regulation mechanisms are involved in berry ripening events and that the developmental timing of these regulatory events is influenced by vine water status.

RESUMO

A vinha (*Vitis vinifera* L.) é uma das mais importantes culturas fruticolas a nível mundial. No velho mundo vitivinícola a vinha é tradicionalmente uma cultura não-regada. Contudo, a introdução da rega começa a torna-se uma prática agronómica vulgarizada com o objectivo de evitar os efeitos negativos de condições prolongadas de deficit hídrico e elevadas temperaturas a que as vinhas estão expostas durante o período de crescimento vegetativo e maturação do bago. Os modelos de alterações climáticas prevêm, particularmente na região Mediterrânea, que o deficit hídrico se torne um dos factores mais limitantes para a produtividade e qualidade da produção vitícola. Paradoxalmente, a imposição de stress hídrico moderado através do uso de estratégias de rega controlada, tem sido uma das ferramentas agronómicas utilizadas pelos viticultores para manipular as características sensoriais da uva sem que se verifiquem perdas significativas de produtividade.

A uva, descrita como um fruto não climactérico, sofre um conjunto de alterações bioquímicas desde o seu desenvolvimento até à sua maturação sobre as quais o nosso conhecimento ainda é limitado, em particular no que diz respeito aos mecanismos moleculares reguladores da iniciação do processo de amadurecimento. À vindima, a qualidade da uva é determinada essencialmente pelo teor em açúcares/ácidos que se acumulam maioritariamente na polpa e pela sua composição em compostos fenólicos, nomeadamente flavonóides que se localizam na película. São estes os principais compostos que irão contribuir para a cor, sabor e aroma do vinho.

Neste contexto, este projecto de doutoramento teve como principal objectivo a obtenção de novos conhecimentos referentes aos mecanismos moleculares involvidos no processo de amadurecimento do bago de uva e a influência da condição hidríca da videira em todo este processo. Este estudo foi realizado com frutos colhidos em diferentes estádios fenológicos, obtidos de videiras da casta Aragonez dada a sua relevância na produção vitícola na Península Ibérica. O efeito da condição hidríca das videiras na regulação de alguns dos principais eventos metabólicos que occorrem durante o amadurecimento da uva foram estudados ao nível do transcriptoma e do

proteoma na película da uva (Capítulos II e III). Foi também avaliado o seu impacto em parâmetros de qualidade como sejam o conteúdo do fruto em antocianinas e açúcares (Capítulos II e III). Dada a relevância das antocianinas como um dos principais flavonóides que se acumulam ao longo do processo de maturação da uva, foi ainda estudada uma proteína da família dos transportadores ABC (do inglês 'ATP-binding cassette'), VvABCC1, potencialmente envolvida no processo de transporte vacuolar das antocianinas sintetizadas no citoplasma (Capítulo IV).

Os resultados obtidos mostraram que o impacto fisiológico do deficit hídrico, classificado como suave a moderado, imposto pela rega controlada (RDI, do inglês 'Regulated-deficit irrigation') ou pela ausência de rega (NI, do inglês 'Non-irrigation'), respectivamente, não foi desfavorável à acumulação de açúcares e antocianinas quando comparado com a condição controlo (FI, do inglês 'Full-irrigation'). Mais ainda, observou-se que a acumulação de açúcares foi antecipada nas condições de deficit hídrico (RDI e NI), enquanto ao nível das antocianinas somente na condição RDI a sua biossíntese foi antecipada. Ao nível transcripcional poucas foram as alterações que poderão ser associadas com esta antecipação nos bagos RDI. Umas destas alterações foi observada no gene *MYBA3*, um factor de transcripção não funcional em videira, que poderá estar envolvido na regulação da síntese de antocianinas através de um mecanismo de competição com os factores de transcripção MYBA funcionais. Este gene foi induzido nas fases iniciais na condição NI. As condições de deficit hídrico tiveram também um impacto ao nível de genes relacionados com o metabolismo das hormonas, como sejam o ácido abcísico e o etileno, hormonas potencialmente envolvidas na regulação do amadurecimento da uva. Os dados recolhidos em relação aos genes envolvidos na síntese do 'açúcar sinal' trealose-6-fosfato sugerem um potencial envolvimento nos mecanismos de amadurecimento da uva. No seu conjunto, os resultados ao nível transcripcional poderão ser explorados num futuro próximo em estudos de funcionalidade de muitos destes genes que se mostraram regulados durante o processo de amadurecimento do bago de uva, mas cuja função se encontra ainda por definir.

Ao nível do proteoma, 74 proteínas foram identicadas e funcionalmente classificadas como maioritariamente associadas ao metabolismo dos hidratos de carbono e aos efeitos do stress. A antecipação da acumulação de açúcares, em particular na condição RDI, foi proposta como um importante factor para as alterações observadas ao nível do proteoma da película da uva. Oito proteínas, que especificamente foram induzidas na condição RDI quando os bagos começaram a acumular açúcares e antocianinas foram propostas como 'marcadores' do ínicio do processo de amadurecimento.

Os resultados ao nível transcripcional e do proteoma sugerem o envolvimento de modificações pós-traducionais na regulação dos mecanismos de maturação, uma vez que alguns dos resultados mais relevantes ao nível das proteínas, como por exemplo os referentes às invertase vacuolar, 'thaumatin-like' e chitinase IV, não foram corroborados ao nível transcripcional.

A caracterização bioquímica da proteína VvABCC1, demonstrou que *in vitro* esta proteína tem a capacidade de transportar para o vacúolo antocianinas glicosiladas mas também conjugados de auxina, sugerindo que transportadores do tipo ABC participam no processo de amadurecimento: na película no transporte de antocianinas e na polpa no transporte de conjugados de auxina.

Os resultados aqui apresentados mostram a complementariedade de estudos ao nível do transcriptoma e do proteoma, sugerindo que mecanismos pós-tradução são importantes para a compreensão da complexidade dos processos regulatórios que envolvem o amadurecimento da uva e que a condição hídrica da videira tem um importante impacto nestes processos.

CONTENTS

Chapter I

General Introduction

1. PLANT RESPONSES TO WATER DEFICIT

Abiotic stress, e.g. drought, high salinity and extreme temperatures, limit crop productivity and play a major role in determining the distribution of plant species across different types of environments (Boyer 1982; Verslues et al. 2006). Global climate change models suggest an increase in the frequency of extreme meteorological events and in aridity, particularly severe in the Iberia Peninsula (IPCC 2008). Climate change has turned agriculture into a more complex activity, with crop performance under sub-optimal conditions being the subject of intense research. The ability to circumvent the negative impacts caused by these environmental constraints brought new challenges to plant research (Chaves and Oliveira 2004).

Most frequently, soil water deficit and high vapour pressure deficit (VPD) are associated with high temperature and high irradiance in semi-arid regions which can lead to oxidative stress in addition to the direct effect of water stress (Wilkinson and Davies 2010). Water stress has been defined as the induction of turgor pressure below the maximal tissue potential pressure (Pugnaire et al. 1999). Plants have developed ecological and physiological strategies to cope with water shortage and can exhibit either *drought escape* or *drought resistance* mechanisms, with resistant plants further classified as *drought avoiders* or *drought tolerators* (Levitt 1972; Bray 1997). Plants can escape drought, for example, by anticipating their reproductive period. *Drought avoiders* maintain water potential despite water deficit, whereas *drought tolerators* withstand water deficit with low tissue water potential (Ingram and Bartels, 1996). Mechanisms such as improved water uptake under stress and the capacity of plant cells to hold acquired water confer drought avoidance. Tolerance mechanisms result from the co-ordination of physiological and biochemical alterations at the cellular and molecular level (Ramanjulu and Bartels 2002) and include among other mechanisms the maintenance of cell turgor by accumulating compatible solutes (Pugnaire et al. 1999; Chaves et al. 2003; Verslues et al. 2006; Harb et al. 2010). This classification, maybe

useful for a systematic analysis, but in practice plants may combine a variety of such response types (Chaves et al. 2003).

1.1. From perception to response

Plant responses are complex and encompass many aspects, including stress sensing and signalling, changes in growth and biomass allocation patterns, water status homeostasis, osmoregulation and detoxification processes (Chaves et al. 2003).

The first step in the regulation of plant's response to stress is its recognition. Soil water deficit can be perceived in drying roots that can propagate chemical and hydraulic signals throughout the whole plant (Davies and Zhang 1991). The evidence for root-derived ABA as a long-distance signal has been obtained from split-root experiments, in which one part of the root system experienced water deficit (Gowing et al. 1990; Wilkinson and Davies, 2002). ABA-precursors, cytokinins and pH variations of xylem can all be involved in the complex signalling of water use regulation at the leaf level (Wilkinson and Davies, 1997, 2002, 2010). Moreover, it seems that a combined action of hormones may exist in root-shoot communication (Wilkinson and Davies 2010). Several studies have indicated hydraulic signals as having a dominant role in the control of stomata in response to decreased soil water availability (Christmann et al. 2007; Rodrigues et al. 2008). However, most evidence suggests that both, chemical and hydraulic signals are important in the stomatal regulation (Comstock 2002). At the whole plant scale, after plant's perception of the stress, one of the first events that occurs in the plant is stomatal closure, as well as leaf and shoot growth inhibition, leading in turn to a reduced net CO_2 assimilation rate, decreased root growth, and finally, if the stress is extreme, induction of leaf senescence and plant death (Passioura 1996). At the cellular level, drought triggers cellular dehydration, which causes osmotic stress (Bartels and Sunkar 2005). Several candidates for sensing osmotic stress have been identified in *Arabidopsis thaliana*, namely ATHK1 (hybrid-type histidine kinase) and Cre1 (cytokinins response 1) (Urao et al. 1999; Reiser et al. 2003). Following stress perception, the signalling transduction cascade will activate the expression of appropriate responses. Stress signalling has been distinguished into ABA-dependent and

ABA-independent pathways (Yamagushi-Shinozaki and Shinozaki 2005; Shinozaki and Yamagushi-Shinozaki 2007). Most of the key genes in these pathways have been identified, such as transcription factors (DREB, CBF, ABF, MYC, MYB), stress-responsive cis-elements (ABRE, DRE) and secondary messengers (Ca^{2+}, reactive oxygen species, salicylic acid). Under drought, these regulatory mechanisms induce downstream alterations in functional genes with the synthesis of several proteins, e.g., aquaporins, vegetative storage proteins, dehydrins, lipid-transfer proteins, proteinase inhibitors or proteins related to the synthesis of osmolytes and soluble sugars (Ramanjulu and Bartels 2002; Bartels and Sunkar 2005; Shinozaki and Yamagushi-Shinozaki 2007).

2. GRAPEVINE

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops worldwide. Apart from their use for wine production, grape berries can also be consumed as fresh or dried fruit, for juice production and, more recently, in nutraceuticals and cosmetics industries. *V. vinifera* is mainly grown in semi-arid ecosystems (Chaves et al. 2010; Lovisolo et al. 2010), to which is well adapted. Yield and berry quality are strongly dependent on vine adaptability to drought. Grapevine is generally classified as a *drought avoider* species (Schultz 2003), mostly due to its large and deep root system and drought avoiding mechanisms, such an efficient stomatal control of transpiration and xylem embolism (Jones 1998; Lovisolo et al. 2008).

In Europe high-quality wine producing areas is traditionally non-irrigated. The foreseen scenario, that in the Mediterranean area more prolonged drought periods and extreme meteorological events such as heat waves will increase, requires agronomical tools that can ensure plant physiological homeostasis and an adequate balance between vegetative and reproductive growth, while preserving yield quality (Chaves et al. 2010).

Under water deficit, stomata closure is among the early grapevine responses; it restricts water loss by transpiration but limits as well carbon assimilation. Reductions in shoot and leaf growth are the first visible sign of vine water deficit (Stevens et al. 1995). Root growth also decreases but to a lesser extent than shoot, which generally promotes an

higher root:shoot ratio and maintains water and nutrient supply to the shoots (Lovisolo et al. 2010). Grapevine vegetative growth is more sensitive to water stress than is reproductive growth. However, this is dependent on the developmental stages of the vine (Keller 2005). Water deficit can significantly reduce yield if it occurs early in the season, particularly when close to and during flower blooming, pollination or fertilization periods (Williams and Matthews 1990). When water stress occurs after fruit set, grapevines can maintain fruit growth and ripening at the expenses of shoot and root stored reserves (Keller 2005).

2.1 Berry ripening

The grape berry consists of a pericarp tissue and seeds (up to four); the pericarp is divided in exocarp (skin), mesocarp (pulp/flesh) and endocarp (Kanellis and Roubelakis-Angelakis 1993). The exocarp cells have an active metabolism, potentially with a regulatory function to the other pericarp tissues (Coombe 1973). Most of the colour, aroma and flavour compounds accumulate in exocarp tissues. The mesocarp accumulates primarily high amounts of organic acids and sugars in the vacuoles; at maturity it accounts to up 90% of the berry weight. The endocarp is the tissue that surrounds seeds and is hardly distinguishable from the mesocarp (Kanellis and Roubelakis-Angelakis 1993).

Grape berry exhibits a double sigmoid growth pattern (Coombe and Hale 1973) as presented in Figure 1: stage I (green or herbaceous phase) is characterized by initial intense cell division and later cell expansion, with berry and seed embryos being completely formed. This stage varies from 45 to 60 days after flowering (Coombe and McCarthy 2000). By the end of stage I chlorophyll is the predominant pigment, berries display an active metabolism and intense tartaric and malic acids accumulation (Kanellis and Roubelakis-Angelakis 1993); stage II (lag growth phase) is characterized by the rapid development of embryos and corresponds to the end of the herbaceous phase of the fruit. The duration of this stage depends on the cultivar, timing of flowering and vine's environment (Coombe 1976); the transition between stages II and III (ripening phase) is called by the French viticulturist's *véraison* and describes in red cultivars the change of

berry skin colour. Overall, stage III lasts 5-8 weeks and is characterized by an acceleration of growth, softening of the berry, accumulation of sugars, anthocyanins (red cultivars), total and free amino acids, flavour and aroma compounds and the decrease in the concentration of organic acids, mainly malic acid, and loss of chlorophyll and phenolic compounds such as tannins (Kanellis and Roubelakis-Angelakis 1993). So, fruit ripening can be described as the integration of biochemical and physiological alterations that occur at the end stage of fruit development and makes the organ edible and attractive to seed-dispersal by animals (Brady 1987; Giovannoni 2001).

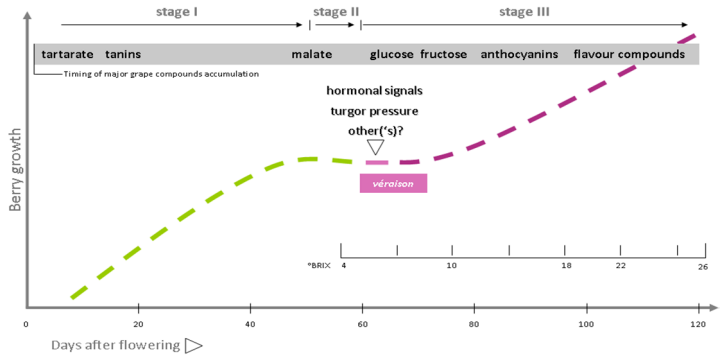

Figure 1 Grape berry development and ripening (adapted from Kennedy 2002).

In recent years the molecular basis of development of fleshy fruits (or non-dehiscent) has received considerable scientific attention, particularly tomato (*Solanum lycopersicum*) (Giovannoni 2001). Accordingly to the physiology of ripening, fruits can either be classified as climacteric or non-climacteric, on the basis of respiration and ethylene biosynthesis rates (Tucker 1993). Climacteric fruits are characterized by a peak of respiration activity and ethylene biosynthesis during ripening, contrarily to non-climacteric fruits. The first group includes fruits such as tomato, banana (*Musa* spp.) and apple (*Malus domestica* L. Borkh). The non-climacteric fruits include grape, strawberry (*Fragaria ananassa* Duch) and orange (*Citrus sinensis* L. Osbeck). Generally, it can be stated that the regulatory mechanisms of fruit ripening events in climacteric-fruits, and

in particularly in tomato, are by far much more clarified than in non-climacteric fruits (Giovannoni 2001; Matas et al. 2009). Hormonal regulation has, for long time, been described as controlling many of the metabolic events that occur at the onset of ripening. While the role of ethylene in climacteric-fruit ripening is well established, the hormonal control of grape berry ripening remains elusive. Ethylene, depending on the time of its application can delay or promote ripening (El-Kereamy et al. 2003; Davies and Böttcher 2009). Auxin treatments delay the gene expression patterns associated with ripening, while abscisic acid (ABA) and brassinosteroids (BRs) play promoting roles (Davies et al. 1997; Jeong et al. 2004; Symons et al. 2006). The available evidence allows us to divide hormones into two groups, those involved in pre-*véraison* grape metabolism – ripening inhibitors (auxins, cytokinins), and those that may have some role in promoting ripening (ABA, ethylene and BRs) (Davies and Böttcher 2009). The control of grape ripening may result from a combination of hormonal signals (Conde et al. 2007; Davies and Böttcher 2009). Also, recently Thomas et al. (2008) observed that turgor pressure (P) in mesocarp cells declines just before the onset of *véraison*. The same authors suggested that P may have a role in many of the physiological and molecular changes that occur at the onset of ripening.

While the biosynthesis of anthocyanin has been extensively described in grape berry (reviewed by Boss and Davies 2009), still little is known about the sequestration of these compounds in the vacuole. However, recently two Multidrug and Toxic Extrusion (MATE) proteins have been implicated in the mediated transport of acylated anthocyanins (Gomez et al. 2009). Also, there has been a long debate as to whether ABC transporters are involved in anthocyanin transport to the vacuole (Klein et al. 2006; Martinoia et al. 2007; Shiratake et al. 2007). The sequestration of anthocyanins in the vacuole is an important process for the survival of the cells because anthocyanins are believed to be toxic (Boss and Davies 2009).

2.2 Impact of Water deficit

Large and dense canopies that result from abundant water availability produce grapes with reduced sugar, high acidity and poor colour accumulation. Moreover, dense

canopies also increase the risk of biotic stress, namely *fungi* attack (Dry and Loveys 1998), further reducing berry quality. On the other extreme, severe water deficit[1] can lead to decreases in both yield and quality as a result of a limited supply of assimilates to the fruits, but as well of an excessive fruit exposure to sunlight (van Leeuwen et al. 2009).

It is generally accepted that mild water deficit[1] is beneficial for fruit and wine composition (Bradvo et al. 1985). An *adequate* water deficit can be induced and maintained by vineyard management techniques such as deficit irrigation strategies. Deficit irrigation aims at a more sustainable use of water, by increasing water use efficiency, while sustaining yield and potentially improving fruit quality (Dry et al. 2001; Chaves et al. 2010). Regulated deficit irrigation (RDI) is one of those strategies that control vegetative and reproductive growth by a deliberate withholding of irrigation during specific periods of the crop cycle, particularly between fruit set and *véraison* (Dry et al. 2001). Water deficit influences grape berry development, metabolism and final composition. Its timing and intensity dictate the extent of alterations occurring in wine colour and flavour. Several studies have focused on the effects of water deficit on the accumulation of the compounds responsible for many organoleptic properties in grapes (Kennedy et al. 2002; Ojeda et al. 2002; Koundouras et al. 2006; Bindon et al. 2007; Santos et al. 2007; reviewed by Chaves et al. 2010). Grape berry quality at harvest is largely dependent on sugar/acids balance but as well on composition of phenolic compounds. The great significance of phenolic compounds in grape concerns their contribution to colour, taste and flavour in fresh fruits and also to the aroma, colour and flavour of wines. For winemaking, harvest dates are usually chosen to obtain a balanced fruit in terms of sweetness, acidity, flavour and phenolic contents. The phenolic compounds that accumulate on grape berries can be divided in non-flavonoids (e.g. stilbenes, volatile phenolics, coutaric and caftaric acids) and flavonoids (e.g. proanthocyanidins, anthocyanins, flavonols) (Adams 2006; reviewed by Conde et al.

[1] According to van Leeuwen et al. (2009) a severe water deficit can be considered when leaf water potential at the pre-dawn (Ψ_{pd}) is lower than -0.8 MPa, whereas a mild stress is observed when Ψ_{pd} varies between -0.3 and -0.5 MPa.

2007) as represented in Figure 2. Proanthocyanidins or condensed tannins are flavan-3-ol oligomers, which represent the most abundant class of phenolics in grape berries (reviewed by Terrier et al. 2009). As they are extracted during winemaking, they contribute to colour stability in red wines, astringency and bitterness (Terrier et al. 2009). Among grape pigments, anthocyanins are the predominant ones in berry skins of red cultivars. Flavonols, such as quercetin can conjugate to anthocyanins, a process called co-pigmentation that reinforces the stability of wine colour (Boulton 2001).

Still controversial is whether under water deficit conditions the increase observed in skin metabolites is influenced by a differential growth of exocarp versus mesocarp (Mathews, Anderson 1998; Ojeda et al. 2001) or, rather from a direct effect on the biosynthetic pathways. In fact, recently Castellarin et al. (2007) showed that water deficit promoted an increased gene expression of several enzymes-associated with the phenylpropanoid pathway.

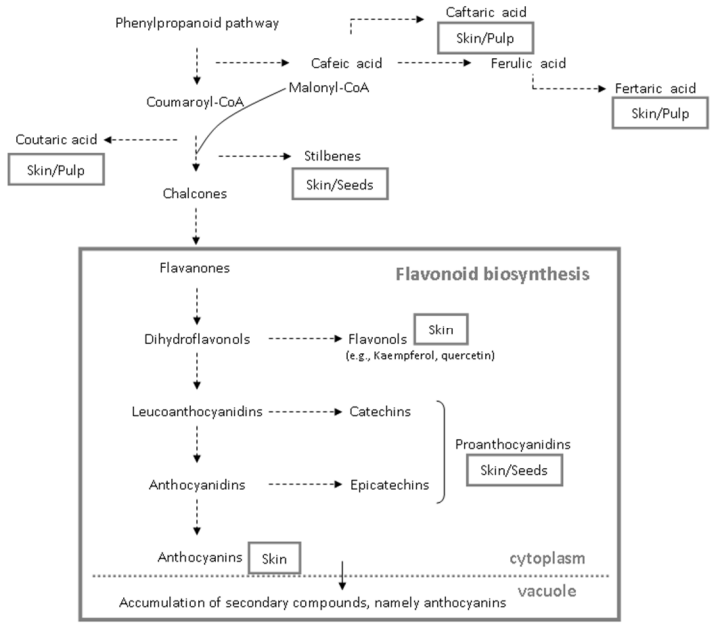

Figure 2 Biochemical pathways of the major phenolic compounds present in grape berries (adapted from Adams 2006; Bogs et al. 2007).

In summary, qualitative differences in berry characteristics produce unique organoleptic characteristics most relevant for the winemaking industry. Still much remain to be learned about the mechanisms underlying the profound events that occur during fruit development and ripening, regardless of the significant progress made over the last years (Conde et al. 2007; Grimplet et al. 2009). The development of high-throughput techniques for analysing grape genome, proteome and metabolome and the release of the grapevine genome sequence (Jaillon et al. 2007; Velasco et al. 2007) resulted in the accumulation of large quantities of biological data that now poses a new challenge to the grape scientific community: to elucidate the true biological meaning of these data (Grimplet et al. 2009).

3. SCOPE OF THIS THESIS

Since grape skin (exocarp) tissue is of outmost importance to the overall grape berry functioning as it protects the inner tissues (mesocarp and endocarp) from external agents, has some potentially regulatory functions and is the site of the synthesis of important metabolites for winemaking, we focussed our main studies on grape berry skin.

The overall goal of this thesis was to obtain new clues on the molecular and biochemical mechanisms that occur during grape berry ripening and how the use of different irrigation strategies can manipulate grape berry metabolism. Moreover, due to anthocyanins relevance in grape berry ripening and the fact that the mechanisms involved in anthocyanins vacuolar sequestration still remain obscure, the hypothesis that ABC-family members could be involved in such process (as suggested in other model systems, like maize) led to its study in grape berry.

The specific questions that were raised include:

1) What were the main metabolic alterations observed during the course of berry ripening?
2) At the transcriptional level which major ripening events were modulated by the different water status conditions?

3) What were the major alterations observed during berry development in what concerns grape skin berry proteome?
4) Was VvABCC1, the closest homologue of ZmMRP3, biochemically able to transport anthocyanins into the vacuole in grape berries?

The findings presented in Chapters II and III provide the identification of the major metabolite trends observed during fruit ripening (four developmental stage: 44, 65, 78 and 98DAF) and the major effects of WD conditions (regulated-deficit irrigation and non-irrigation) as compared to full-irrigated conditions. This was mainly achieved through NMR (sugars and organic acids) and biochemical (anthocyanins, total phenols) determinations. The genome-wide analysis of transcript abundance (through the use of microarray technology) showed the modulation of several hormonal and non-hormonal regulatory mechanisms, but also provided some clues for further functional analysis, in what concerns for instance some transcriptional factors or trehalose 6-phosphate metabolism for example (Chapter II). The proteome data allowed us to identify the major proteins present in grape skins during fruit ripening (2DE-MS/MS analysis). Moreover, some alterations observed at the proteome level suggested that some proteins may be under the regulatory control of signals such as ABA and sugars. Post-translational modifications may also be part of the complex suite of events that occur during grape berry maturation.

The experimental evidence provided in Chapter IV showed that VvABCC1 is able, *in vitro*, to transport anthocyanins into vacuoles. Moreover, we further suggest that VvABCC1 may also be involved in other events that occur during ripening, since it was also shown that it is able to transport auxins conjugates.

Finally, chapter V reviews the most updated information in what regards grapevine performance under deficit irrigation conditions.

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ChapterII

Genome-wide analysis of grape berry ripening under water deficit conditions

Version of the manuscript entitled: *Genome-wide analysis of grape berry ripening under water deficit conditions*

Rita Francisco[1], Diego Lijavetzky[2], Gema Bravo[2], Cândido Pinto Ricardo[1,3], José Miguel Martinez Zapater[2], Maria Manuela Chaves [1,3]

[1]Instituto de Tecnologia Química e Biológica, Portugal; [2] Centro Nacional de Biotecnología (CNB), Spain; [3]Instituto Superior de Agronomia, Portugal.

In preparation

R Francisco declares that have actively contributed to the experimental design, grape sampling, RNA extraction, qRT-PCR validation data, bioinformatic analysis, data interpretation and manuscript writing.

ABSTRACT

Grape berry ripening is a complex process that involves an intricate suite of physiological, biochemical and molecular events that culminates in an edible fruit, characterized by e.g. high amounts of sugars and phenolic compounds. Environmental factors, such as water deficit (WD) conditions are known to influence this process. In order to get new insights on the transcriptional network involved in berry ripening and to evaluate the impact that contrasting water status conditions have on such process, mRNA profiling of grape berry skins at four developmental stages: from green stage (44DAF), véraison (65DAF) to mature berries (78 and 98DAF) and under three irrigation conditions [full-irrigated (FI), regulated-deficit irrigated (RDI) and non-irrigated (NI)] was performed using a new custom made GrapeGen Affymetrix GeneChip. Anthocyanins and sugars were also profiled during grape maturation. WD had an impact on both sugars and anthocyanins accumulation. Over the course of berry ripening 3740, 4817 and 4200 genes showed at least a two-fold change at one or more berry stages in FI, RDI and NI berries, respectively. mRNA profiles from RDI berries were those than in general presented singular expression profiles when compared to both FI and RDI. The high number of differentially expressed transcription factors confirms that grape ripening is under a tight transcriptional control. The expression profiles of some of the most relevant ripening-related events were presented. Moreover, the expression of ABA-related transcripts suggested that ABA may be modulated during berry ripening by a dynamic balance between biosynthesis and catabolism events. Peptide signalling, mediated by Rapid Alkalinization factors and Phytosulfokines may also be involved in grape berry development and ripening events. Our results showed that WD had an impact on transcripts e.g. related to hormonal signalling pathways, flavonoids, sugars, cell wall metabolism and aquaporins.

Keywords: Vitis vinifera, grape berry, ripening, water deficit, gene expression, microarrays

1. Introduction

Growing grapevines under restricted water supply has long been regarded as an agronomical tool for manipulating berry sensory characteristics. Overall, it can be summarized that plant water status affects grape berry quality at harvest, which is largely dependent on sugar/acids balance as well on compounds responsible for the organoleptic properties of grapes. Several physiological studies have focused on the effects of water deficit (WD) on the accumulation of these compounds (Kennedy et al. 2002; Roby et al. 2004; Bindon et al. 2007; Santos et al. 2007). However, our understanding of such effects at the molecular level is still emerging. Grimplet and co-workers (2007) apart from an extensive catalogue of grape tissue-specific gene expression profiles also presented a transcriptional analysis of WD effects on grape berry at the end of the ripening stage. In this study, well watered plants (watered from berry setting on) were compared with non-irrigated plants. It was observed that WD had an effect on mRNA expression patterns particularly associated with cell wall, sugars but the most profound alterations were observed on ethylene, auxin and abscisic acid metabolism. Castellarin et al. (2007a) studied in Merlot the effect of WD in the flavonoid pathway, particularly the transcriptional regulation of anthocyanins biosynthesis. It was observed that anthocyanins biosynthesis was enhanced, but none of the collected molecular evidence suggested that ABA or sugar- signalling components were altered. The same research team (Castellarin et al. 2007b) studied the effect of early WD (no irrigation from fruit set until the end of véraison) versus late season WD (no irrigation from the end of véraison on) on the expression of genes of the flavonoid pathway, in Cabernet Sauvignon grapevines. It was observed that early WD hastened the onset of sugars and anthocyanin accumulation, but at harvest no significant increase in anthocyanins content was observed. Deluc et al. (2009) integrated transcript and metabolite profiling data of Chardonnay (a white-wine variety) and Cabernet Sauvignon responses to WD. The authors observed that WD enhanced photoprotection mechanisms at Chardonnay berries, whereas an induction of ABA, proline, sugars and anthocyanins content was observed in Cabernet berries. It was further suggested that

these WD alterations in Cabernet were under ABA-signalling activating mechanisms. Overall, these results emphasise that the effects of WD are dependent on the variety but as well on the environmental conditions that occur during the growing season (Dry and Loveys 1998, Santos et al. 2005).

Grape berry development and ripening is a complex process of physiological and biochemical alterations (Kanellis and Roubelakis-Angelakis 1993; Coombe and McCarthy 2000). Berry growth follows a double sigmoid growth curve (Coombe 1976). Stages I and III of growth are separated by a lag phase (stage II) known as véraison. During stage I, imported carbohydrates are used for seed development, cell proliferation and expansion, and synthesis of organic acids. The second phase of berry growth (stage III, ripening phase) is mostly characterized by berry softening, sugars and anthocyanins accumulation. Although the genetic control of ripening is not fully understood, hormonal regulation and turgor pressure have been described as controlling many of the metabolic events that occur at the onset of ripening, beginning of stage III (see review Davies and Böttcher 2009; Thomas et al. 2008).

The first high-throughput transcripts profiling in grape berry (Deluc et al. 2007; Pilati et al. 2007; Lund et al. 2008; Zenoni et al. 2010) provided a comprehensive picture of gene regulation but also illustrated the complexity of the alterations that occur during berry development and ripening. Much progress is nowadays expected, since the available grape genome (Jaillon et al. 2007; Velasco et al. 2007) is now the framework for analysing Vitis gene families such as transcription factors (Matus et al. 2008; Díaz-Riquelme et al. 2009; Licausi et al. 2010), sugar transporters (Afoufa-Bastien et al. 2010) or pathogenesis-related proteins (Lebel et al. 2010; Li et al. 2011).

Therefore, to uncover the complex biochemical pathways that occur during berry development and ripening is regarded of outmost importance for viticulture ameliorations. The present work describes a transcriptomic study of field-grown grape berries (Vitis vinifera var. Aragonez) aiming at further understanding the impact of different grapevine water status on fruit ripening: full-irrigated, regulated-deficit irrigated and non-irrigated grapevines were studied. The specific goals of this work were

to (i) characterize the major transcriptional alterations that occur during fruit ripening in berry exocarp tissues, due to the relevance of this tissue in what concerns grape organoleptic properties and (ii) and evaluate the impact of WD on the major ripening events at the transcriptional level, including any alteration in the timing of occurrence.

2. Results and Discussion

2.1. Microarray analysis

Genome wide analysis of gene expression variation during grape berry development and ripening was assessed using the Grapegen Affymetrix GeneChip™ (Lijavetzky et al. *in preparation*). Four time points (from green to mature berries) were selected for sampling: 44, 65, 78 and 98 DAF under three irrigation strategies: full-irrigated (FI, with ψpd ranging from -0.22 to -0.43 MPa throughout the growing cycle), regulated-deficit irrigatied (RDI, with ψpd ranging from -0.41 to -0.75 MPa) and non-irrigated (NI, ψpd ranging from -0.65 to -0.86 MPa) but rain-fed vines.

Datasets from each irrigation strategy were separately analysed. From the 23096 probesets represented on the array an overall mean call of 75.1% ± 1.1 was calculated when considered all the studied conditions. Analysis of variance (ANOVA $p<0.05$, false discovery rate 0.05) indicated that 12325 probesets were differentially expressed at one or more berry stages under FI conditions and from those 3740 displayed at least a two-fold change. Under RDI from a core of 14448 differentially expressed genes, 4817 exhibit at least a two-fold change and finally, under NI conditions 12229 probesets were differentially expressed and from those 4200 showed a two-fold change as a minimum. Only the set of genes that displayed a two-fold change ($|FC|>2$) were considered for further analysis (Additional file 1). Pearson's correlation distance based on the gene expression profiles was used to perform a K-means cluster analysis. Transcripts were divided into 20 clusters (Additional file 2). In all conditions the majority of the clusters represented transcripts positively modulated by the ripening events (52, 55 and 61% under FI, NI and RDI respectively). Grapegen Affymetrix GeneChip™ was functionally classified into 10 major functional classes (Lijavetzky et al. *in preparation*): 'Cellular

process', 'Development', 'Diverse functions', 'Metabolism', 'Regulation overview', 'Response to stimulus', 'Signalling', 'Transport overview', 'Unknown' and 'Xenoprotein' (Additional file 1).

The results obtained with the microarray analysis were validated with quantitative real-time RT-PCR (qRT-PCR) assays on 11 cDNA sequences using gene-specific primers (Additional file 3) based on the corresponding GrapeGen GeneChip™ probeset sequences. The qRT-PCR profiles were analysed on three biological replicates of FI berries during fruit development (44 to 98DAF). Linear regression analysis displayed significant correlations for 9 out of 11 genes evaluated (Additional file 3).

2.2. Functional comparison of differentially expressed genes during fruit ripening and under different plant water status conditions

We have chosen to analyse the transcriptional profile of the major processes that have been described as ripening associated, namely flavonoids biosynthetic pathway, sugars metabolism, cell wall metabolism, but as well some the putatively regulators of these events (e.g. hormonal and non-hormonal signalling pathways, transcription factors). All information regarding the differentially expressed genes under the different studied conditions are presented in Additional file 1.

2.2.1.*Hormones metabolism*

The role of several hormones in grape development and ripening has been described by several authors (Tucker 1993; Davies and Böttcher 2009). In the next section we present and discuss the major results obtained when studying the effects of different plant water status on the metabolism of grape skins hormones during fruit ripening.

2.2.1.1. Abscisic acid

The involvement of abscisic acid (ABA) in grape berry ripening events has been confirmed by ABA accumulation patterns (Davies et al. 1997; Wheeler et al. 2009) as well at transcript and protein levels (Deluc et al. 2007; Pilati et al 2007; Giribaldi et al 2010). ABA concentration has been reported to be higher in the skin than in the flesh, and a sharp increase of ABA concentration is observed around *véraison*, which is

followed by a decrease during berry maturation (Coombe and Hale 1973, Davies and Böttcher 2009). However, our understanding of ABA metabolism and signalling during grape berry ripening is still very poor (Davies and Böttcher 2009).

We observed that *9-cis-epoxycarotenoid dioxygenase* (*NCED*) and *zeaxanthin epoxidase* (*ZEP*), which codify for enzymes involved in ABA biosynthesis, where differentially expressed during fruit development. *ZEP* transcripts under RDI conditions[1] decreased from 44DAF onwards. *NCED* transcripts were also modulated by WD conditions (Figure 1A-C). Two *NCED* genes with contrasting profiles also were identified; both transcripts with similar relative abundance. *NCED* (VVTU6502_at) was negatively modulated by ripening. Indeed, at 44DAF the highest expression of this gene was detected under NI conditions. On the other hand, *NCED* (VVTU8254_at) was enhanced from 44DAF onwards. Under NI the up-regulation was more intense (5-fold increase against a 2-fold under FI and RDI). Moreover, under RDI conditions, *NCED2* (VVTU17555_s_at) was also differentially expressed showing transient decreases at 65 and 98DAF. Wheeler et al. (2009) showed that the expression patterns of *NCED* and *ZEP* do not closely correlate with ABA accumulation that occurs during berry development. This contradicts Deluc et al. (2009) who proposed that ABA concentration in berries might be primarily regulated by *NCED1*. Recently, the so-called 'core ABA pathway' has been defined as including three types of proteins: the family of PYR/PYL/RCAR ABA receptors, the clade A of protein phosphatases type 2C (PP2Cs) and three ABA-activated protein kinases from the sucrose non-fermenting1-related subfamily 2 (SnRK2.2, 2.3, 2.6; Cutler et al. 2010). When ABA increase in the plant cells it leads to the PYR/PYL/RCAR receptor-mediated inhibition of the PP2C activity, which results in the activation of the SnRK2s and ultimately the activation of the ABA signalling pathway (Umezawa et al. 2010). We found that *phosphatases type 2C* (*PP2Cs*) were regulated during fruit ripening: *ABI1*, *AHG3-PP2CA* and *FSPP2C2*. We observed that *ABI1* showed highest expression at 65 and 98DAF under FI and NI whereas under RDI it was positively

[1] Under FI conditions *ZEP* was differentially expressed, but excluded from the present analysis since |FC|<2

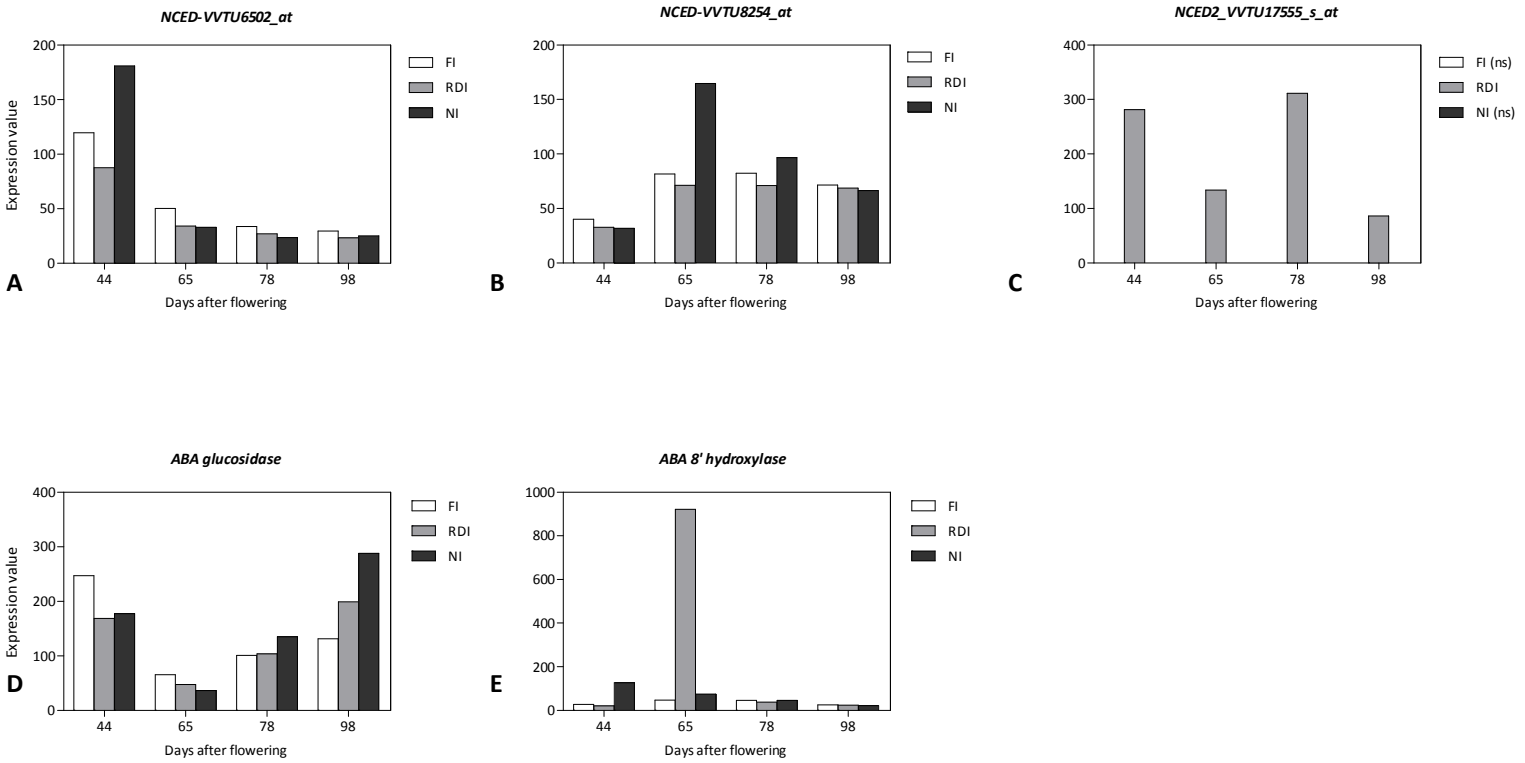

Figure 1 ABA metabolism. Expression profiles of genes involved in ABA biosynthesis: **A-C** [*9-cis-epoxycaroteonoid dioxygenase (NCED)*]; ABA homeostasis mechanisms: **D-E** (*ABA glucosidase* and *ABA 8'hydroxylase*) during fruit ripening under full-irrigated (FI), regulated-deficit irrigated (RDI) and non-irrigated (NI). Bars represent the average of transcripts expression value of three biological replicates. Only differentially expressed transcripts (ANOVA $p<0.05$, FDR =0.05) and whose $|FC|>2$ at one or more berry stages were considered in the present study; (ns), non significant alteration; 'VVTU' represents probeset ID.

modulated until 78DAF, after which its expression decreased. *AHG3-PP2CA*, which showed the highest relative expression of all *PP2C*, was positively modulated by ripening. *FSPP2C2* was only differentially expressed under RDI, showing a decrease after 65DAF. At the protein level, Giribaldi et al. (2010) identified a PP2C protein similar to AtABI1, which was repressed at *véraison* after ABA treatment. Overall, we observed that *PP2Cs* are induced during fruit ripening, what may indirectly correlate with ABA decrease that is observed in berry skin during berry maturation. The identified receptors *PYL9/RCAR* decreased during fruit ripening, but their profile was influenced by the vines water status. Under both FI and NI its repression was observed from 44DAF on, whereas a steady expression was observed under RDI until 65DAF, after which its decrease was observed. However, the highest relative expression was observed under NI at 44DAF. *PYL8/RCAR* was only differentially expressed under WD conditions (RDI and NI); it was repressed at 65DAF after which a steady expression was observed. The gene *KEG* (*KEEP ON GOING*) was also identified. It was positively modulated during fruit ripening under RDI and NI[2]. KEG proteins are capable of mediating ubiquitylation. In *Arabidopsis*, KEG was proven to have a central role in ABA signalling during post-germination development in *Arabidopsis* seedlings (Stone et al. 2006). ABA can either be degraded, through the irreversible pathway starting with ABA 8'-hydroxylation, catalysed by the cytochrome P450 CYP707A family or stored as conjugated forms (Nambara et al. 2005). ABA can be inactivated by its conjugation with glucose to form ABA glucose ester (ABA-GE; Xu et al. 2002); its deconjugation is catalysed by β-glucosidase. We found that *ABA glucosidase* showed a transient decrease around 65DAF, after which it steadily increased up to later stages of berry maturation. This trend was observed in all conditions, although at 78 and 98DAF the higher relative expression was observed under RDI and NI (Figure 1D). Sun et al. (2010) observed also in grape berries that *ABA glucosidase* remained high from *véraison* onwards. Lee et al. (2006) proposed in *Arabidopsis* that the activation of inactive ABA pools by a β-glucosidase is a mechanism by which cells rapidly adjust ABA levels and respond to changing environmental cues. Under RDI *ABA 8'-*

[2] Under FI *KEG* was also differentially expressed but excluded from this analysis due to |FC|<2

hydroxylase CYP707A1 showed a 44-fold increase at 65DAF, after which a dramatic decline was observed (Figure 1E). In sweet cherry (*Prunus avium* L.) the expression analysis of *ABA 8'-hydroxylase* during fruit maturation suggested that endogenous ABA content are regulated by a dynamic balance between biosynthesis and catabolism, which are respectively regulated by NCED1 and ABA 8'-hydroxylase CYP707A (Ren et al. 2010). Indeed, the fact that this gene under FI or NI showed a relative low expression in all sampling dates and its dramatic transient expression at 65DAF, when RDI berries already accumulate sugars and anthocyanins (see sections 2.2.4 and 2.2.5), suggests that ABA 8'-hydroxylase may be involved in the ripening events. As referred before, in grape berries the correlation between *NCED1* and ABA content is contradictory (Deluc et al. 2009; Wheeler et al. 2009), conjecturing that more complex regulatory mechanisms are involved in ABA accumulation, and may be modulated by a dynamic balance between biosynthesis and catabolism events. More, since *NCED* genes were induced by ABA (Sun et al. 2010), it is still not clear the origin of initial increases of ABA in berry tissues, if resulting from in *situ* biosynthesis or if this increase results from translocated ABA from other grapevine organs (Castellarin et al. 2011).

Several *HVA22* described as '*ABA- and stress-inducible*' genes were identified: *HVA22A*, *HVA22C* and *HVA22E*. *HVA22E* increased during the ripening stage in all conditions. *HVA22A* and *HVA22C* showed to be differentially regulated under RDI as compared to both NI and FI. In RDI, a peak of their expression was observed at 65DAF, followed by a decrease thereafter, whereas under NI and FI *HVA22A* and *HVA22C* decreased already at 44DAF. *ASR2* (*ABA, stress, and ripening-induced*) showed a down-regulation at 65DAF, after which a slight increase was detected in RDI and NI. One member of *ASR* family (*ASR1*) was shown to transcriptionally regulate the hexose transporter *VvHT1* (Atanassova et al. 2003; Cakir et al. 2003).

Overall, our understanding of ABA regulation during fruit ripening is still emerging; the functional characterization of most of all the above described ABA-regulators will for sure contribute to reduce this lack of knowledge. Still we observed that WD induced

alterations in ABA metabolism, namely in what concerns transcripts associated with ABA biosynthesis and ABA homeostasis.

2.2.1.2. Ethylene

The role of ethylene in grape berry development is still the subject of some controversy. Although grape berry is a non-climacteric fruit, the evidence suggests that a minor, transient increase in endogenous ethylene may occur just before *véraison* (Chervin et al. 2004; Davies and Böttcher 2009; Tornielli et al. *in press*). The exogenous application of ethylene was proven to have an effect at the transcriptional level namely transcripts involved in anthocyanins biosynthesis (reviewed by Davies and Böttcher 2009).

In the present study 18, 22 and 17 differentially expressed genes related to ethylene metabolism were identified during fruit development under FI, RDI and NI, respectively. The transcripts involved in ethylene biosynthesis were modulated both by fruit ripening and WD conditions. *S-adenosylmethionine synthetase* (*SAM*) was induced in general at post-*véraison* in all conditions. Under WD conditions the highest relative expression of *SAM* (VVTU18255_s_at) was observed with a peak of its expression occurring 65DAF. Since SAM also participates in the synthesis of other compounds namely polyamines, this observation does not necessarily mean that ethylene biosynthesis is being favoured. *ACS* (*1-aminocyclopropane-1-carboxylate synthase*; VVTU6382_at) was repressed in all conditions from 44DAF onwards (Figure 2A). Several *ACO*s (*1-aminocyclopropane-1-carboxylic acid oxidases*) were differentially expressed during fruit ripening, the majority of them were found to be negatively modulated by ripening (Figure 2B-D). Still, one *ACO* (VVTU2507_s_at) showed under RDI a transient increase at 65DAF, when RDI berries already started to accumulate hexoses and anthocyanins. Presumably a transient increase of endogenous ethylene occurs around *véraison* with the concomitant rise of ACO activity (Chervin et al. 2004). The fact that we observed different *ACO* expression profiles may be the result of differential regulation of grape *ACO* genes. Likewise, in tomato *ACO* gene expression patterns in ripening fruits showed that each *ACO* is highly regulated, with transcripts of individual members accumulating to varying degrees at distinct developmental stages (Barry et al. 1996).

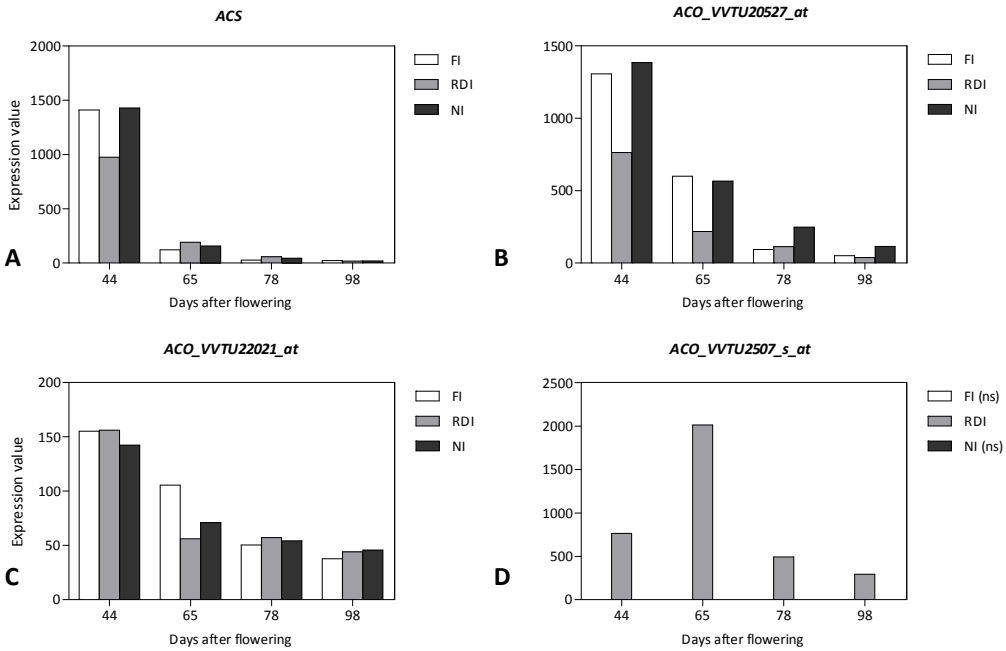

Figure 2 Ethylene biosynthesis. Expression profiles of transcripts involved in ethylene biosynthesis that were modulated during ripening and by FI, RDI and NI: **A**) *ACS* (*1-aminocyclopropane-1-carboxylate synthase*); **B-D**) *ACOs* (*1-aminocyclopropane-1-carboxylic acid oxidase*). (ns), non-siginificant alteration.

Ethylene receptors are the first component in ethylene signalling. We found that *ETR2* (*ethylene receptor 2*) was expressed during fruit ripening and was responsive to WD. Under RDI it was up-regulated at 65DAF, whereas under NI the peak of expression was detected earlier at 44DAF, thereby suggesting a finely tuned regulation via water status conditions. Additionally, *ETR2* was relatively more expressed under RDI throughout berry maturation as compared to NI berries. Both Sun et al. (2010) and Chervin and Deluc (2010) observed a peak of expression of *VvETR2* just before véraison concomitantly to the internal ethylene peak. Ethylene receptors act as negative regulators through CTR1 (CONSTITUTIVE TRIPLE RESPONSE1). CTR1 is key protein that activates the downstream ethylene signal cascade when all receptors are saturated with ethylene (Yoo et al. 2009). We observed that *CTR1* showed a differentially expression pattern during fruit ripening under the different water status conditions. Under FI it was

up-regulated post 65DAF. Under NI a transient up-regulation was observed at 65DAF and finally under RDI it was repressed from 65DAF onwards. This gene was also proven to be associated with ripening events in grape berries where a transient expression was observed pre-véraison (Sun et al. 2010). However, Chervin and Deluc (2010) observed that Vv*CTR1* expression was constant throughout ripening stage. *ERF1* (*Ethylene response factor*) showed a 17, 34 and 24-fold increase from 44 to 65DAF under FI, RDI and NI, respectively, after which it maintained a steady expression throughout berry maturation. The other identified *ERFs* showed a relative lower expression as compared to *ERF1* and were negatively modulated by ripening.

The information about the role of ethylene during grape berry ripening is still fragmentary. Much of the evidence supporting its involvement in ripening is still correlative (Davies and Böttcher 2009). Moreover, it cannot be excluded that some of the alterations observed at the expression of ethylene-related genes may also be associated for example to grape berry responses to abiotic/biotic stressful conditions. Nevertheless, we observed that ethylene sensing/regulatory mechanisms are activated during berry ripening and to a certain extent are modulated by WD.

2.2.1.3. Auxins

Original reports in grape berries described that auxin content is high early in development, reaching its maximum at the end of growth phase II and declining thereafter (Coombe and Hale 1973). In fact, we also observed that the free IAA content is significantly reduced from *véraison* onwards (Francisco et al. unpublished results; see chapter IV).

Numerous genes related to auxin metabolism were identified in our study; their expression profile suggests that auxins may be a part of several processes taking place throughout fruit development and ripening, as already suggested by Deluc et al. (2007) results. We observed that 36, 44 and 36 transcripts were differentially expressed under FI, RDI and NI conditions, respectively. *Indole-3-acetic acid-amido synthetase* (*GH3.8*; VVTU3560_at) was negatively modulated during fruit ripening in all conditions, but this modulation was anticipated by FI and NI conditions, where its relative expression

decreased from 44 DAF onwards, whereas under RDI a peak was observed at 65DAF after which it significantly decreased over the course of berry maturation (Figure 3A). Recently, the potential role of GH3s in endogenous IAA inactivation through the irreversible formation of IAA–Asp conjugate was proposed in grapes and associated with the ripening events (Böttcher et al. 2010, 2011). Moreover, RDI and NI induced the expression at 65DAF of several *auxin efflux carriers* (VVTU34750_s_at; VVTU35909_s_at; VVTU7647_at) whereas under FI the most highly relative expressed transcript (VVTU34750_s_at) was negatively modulated from 44DAF onwards; under NI an *auxin efflux carrier* (VVTU34750_s_at) showed an up-regulation at 65DAF, after which a 7-fold reduction was observed (Figure 3B-D). Overall, under WD conditions a general pattern of an intense up-regulation at 65DAF was observed. *Auxin response factors* (*ARF*) and *AUX/IAA* genes were on the other hand more abundant before *véraison* stage in all conditions (e.g. VvTU6022_at), with the exception of *IAA19* (VVTU3361_at) that was positively modulated during berry ripening in all studied conditions (Figure 3E-G). Kohno et al. (2011) detected a similar expression pattern of this gene in Cabernet berries but still evidence is needed to fully determine the precise biological function of this gene during fruit development in grapevine (Kohno et al. 2011).

In summary, several transcripts related with auxin were differentially expressed throughout berry ripening what suggests that auxin may also have a role during grape ripening, as observed in other fruits (Trainotti et al. 2007). Also, we observed that WD had an effect at *auxin efflux carriers* and *GH3.8* suggesting that auxin homeostasis is being altered by WD effects. But, until the precise role of auxins as regulators of grape berry development and ripening is achieved the interpretation of these data is still limited.

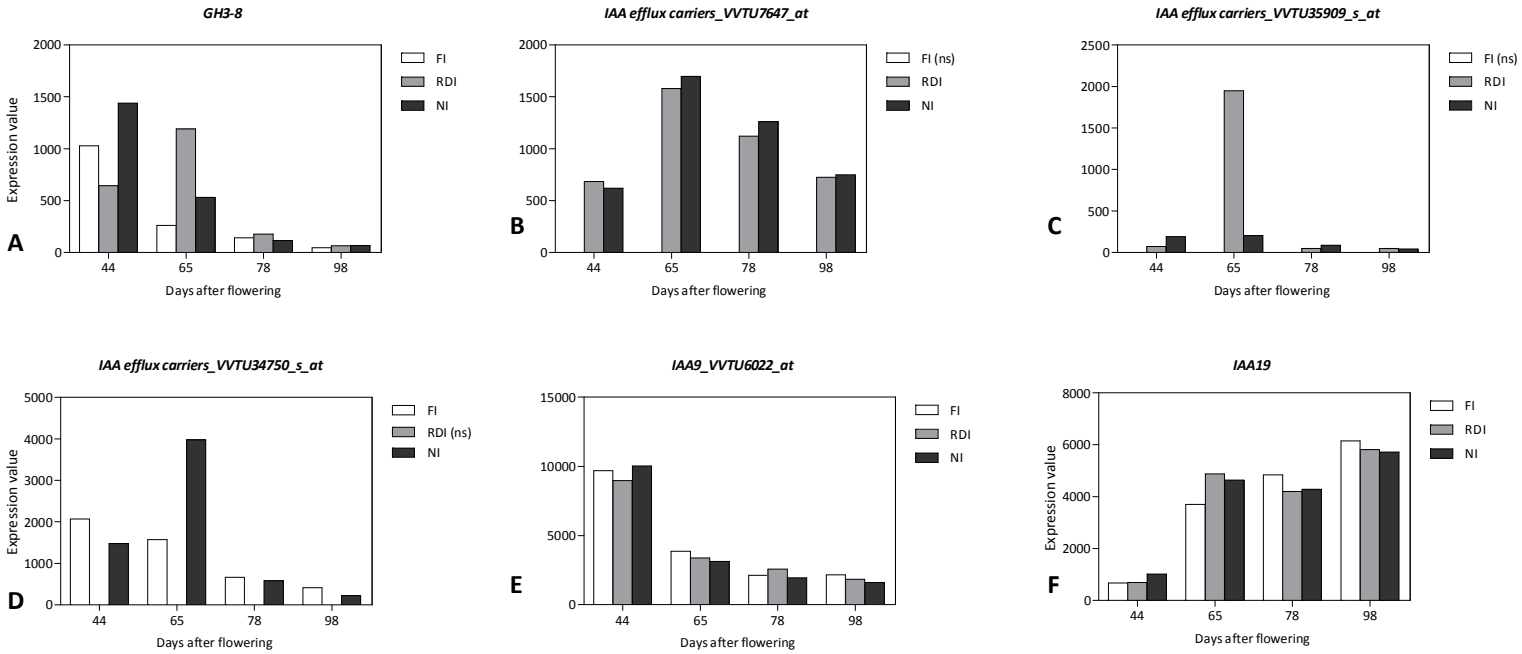

Figure 3 Auxins metabolism. Expression profile of several transcripts involved in auxins regulation during fruit ripening and its modulation by vines water status conditions: **A**) *indole-3-acetic-amido synthetase* (*GH3-8*); **B-D**) *IAA efflux carriers*; **E**) *IAA9* anf **F**) *IAA19;* (ns), non-significant alteration.

2.2.1.4. Brassinosteroids

Only recently brassinosteroids (BRs) emerged as a potential regulator of grape berry ripening since a sharply increase around *véraison* in grape berries was observed (Symons et al. 2006). In our analysis we found several genes related to BRs regulation. *BRI1* (*BRASSINOSTEROID INSENSITIVE 1),* which is a receptor that plays a role in BRs signalling, was repressed from 44DAF onwards under RDI and NI[3]. Interestingly, the relative expression of *BRI1* was as at least 12-fold higher (maximum 24-fold at 78DAF) under RDI conditions (Figure 4A). Symons and colleagues (2006) observed that this gene is constitutively expressed, and a transient peak was observed around *véraison*. *BAK1* (*BRI1-ASSOCIATED RECEPTOR KINASE*) transcripts were negatively modulated by ripening in all conditions (Figure 4B-D). *BZR1* (*BRASSINAZOLE-RESISTANT 1*), which is a positive transcriptional regulator of BRs signalling pathway, was induced at 65DAF under RDI, decreasing thereafter. Remarkably, we observed that *BIN2* (*BRASSINOSTEROID-INSENSITIVE* 2), the negative regulator of BZR1, was constitutively expressed during all grape berry stages studied. The integration of the presented data suggests that BRs metabolism is altered by WD conditions. Symon et al. (2006) proposed that BRs may promote an increase in berry size, at least in part, by regulating cell wall-modifying enzymes expression, namely xyloglucan endotransglycosylase.

2.2.1.5. Cytokinins

Cytokinins (CK) are viewed as grape berry ripening inhibitors, thought to be involved in berry set and growth promotion (Davies and Böttcher 2009). But still much less is known about the role of cytokinins on berry development.

In the present study transcripts related with cytokinins homeostasis, transport and cytokinins-mediated signalling pathway were identified as differentially expressed during fruit development. The majority of these transcripts were related to cytokinins homeostasis, either reversible conjugation or through irreversible inactivation (Auer 1997). Interestingly, *cytokinin dehydrogenase 5 precursor* (VVTU3125_at) and *cytokinin*

[3] Under FI *BRI1* was also differentially expressed during fruit ripening but |FC|<2; moreover the same trend was observed as compared to WD conditions.

dehydrogenase 7 (VVTU9094_s_at) were negatively modulated from 44DAF in all water status conditions. The genes are involved in the irreversible inactivation of cytokinins. Whereas transcripts involved in reversible inactivation (by glycosylation) of cytokinins such as *cytokinin-O-glucosyltransferases* were positively modulated by ripening and also by WD. Overall these results suggests that CK metabolism is active during fruit ripening, but still further evidence of CK exact role in grape berry ripening is required.

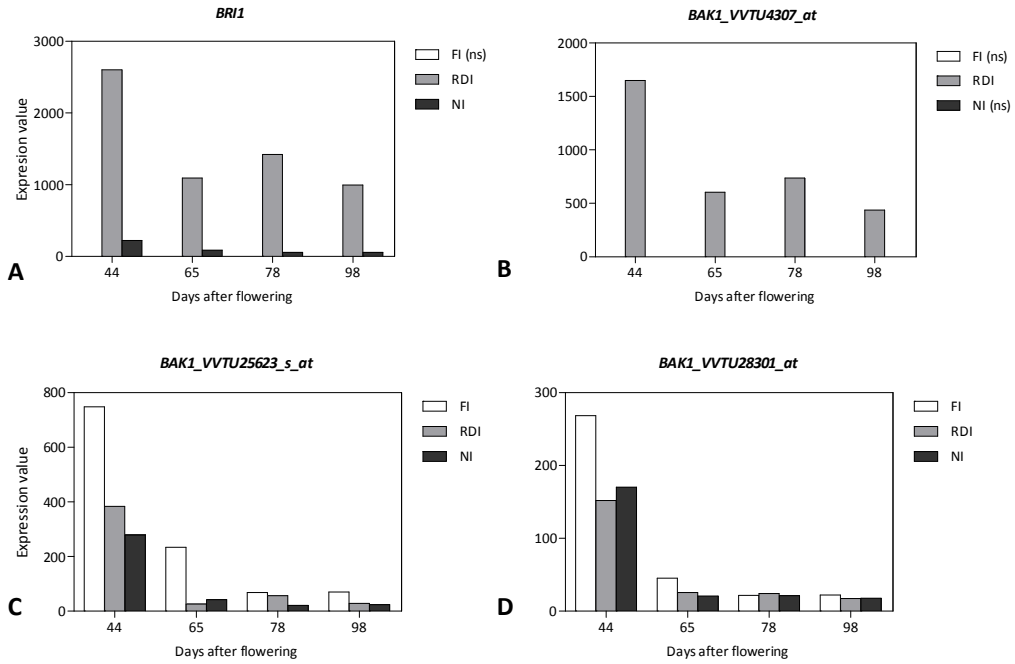

Figure 4 Brassinosteroids regulation. The expression profiles of transcripts related with BRs signal transduction that were differentially expressed during fruit ripening and that were modulated by water status conditions: **A**) *BRI1* (*BRASSINOSTEROID INSENSITIVE 1*); **B-D**) *BAK1* (*BRI1-ASSOCIATED RECEPTOR KINASE*). (ns), non-siginificant alteration.

2.2.1.6. Giberellins

Our understanding about giberellins (GA) role during grape ripening is still very poor, although GA is considered a regulator of growth and development (Davies and Böttcher 2009). We identified only few genes differentially regulated during grape ripening. Interestingly, the transcript abundance of *GID1L1* and *GID1L2* (*Gibberellin receptor*) increased during fruit ripening, in agreement with Deluc et al. (2007). Also *SLY1*

(*SLEEPY1*) a positive regulator of GA increased during fruit ripening but no WD effect was observed.

2.2.2.*Signalling pathways*

2.2.2.1.*Peptide signalling*

RALFs (Rapid Alkalinization factor) are small peptides that initially were isolated in tobacco and induced the rapid alkalinization in cell suspension; they also inhibit root growth in tomato and Arabidopsis seedlings (Pearce et al 2001). We found that several *RALFs* were ripening regulated in our study. *RALF34* was highly expressed and a similar expression pattern was observed in all three water status conditions: RALF34 was up-regulated at 65DAF, after which it decreased until 98DAF. The most intense up-regulation was observed under RDI conditions. Recently, Martínez-Esteso et al. (2010) identified one RALF peptide that accumulated at late ripening stages in grape berry skin.

Three *phytosulfokines* (*PSK1*, *PSK2*, *PSK4*) where found negatively modulated during fruit ripening from 44DAF on. No significant WD effect was observed. The available data indicate that PSK has a role related to cell division and development, but its mechanism of action and the *in vivo* PSK role is still to be determined (Ryan et al. 2002; Matsubayashi and Sakagami 2006).

Peptide signalling regulates a variety of developmental processes and environmental responses in plants (Matsubayashi and Sakagami 2006) and may also be involved in fruit development and ripening, but further evidence is needed to substantiate this assumption.

2.2.2.2.*Other signalling pathways*

Several transcription factors associated with circadian clock were shown to be differentially regulated during fruit ripening, and will later on be discussed. In accordance, other components of the circadian clock signalling were also identified as modulated during fruit ripening, namely *pseudo-response regulator 7* (*APRR7*), *APRR9* and *ZEITLUPE* (*ZTL*; also called *ADAGIO1*). The circadian clock regulates diverse aspects of plant growth and development and also promotes plant fitness (Harmer 2009).

Although no study has been made in what regards fruit ripening, it is predicted that circadian system acts as a signal integrator, interacting with many other signalling networks (namely hormones, temperature, light) to restrict plant responses to environmental cues to the most appropriate time of the day (Harmer 2009).

Also numerous signalling factors such as calcium sensors/signalling, 14-3-3, G-protein signalling, phosphatidylinositol signalling and protein kinases-signalling mediators were transcriptionally associated with fruit development and ripening.

2.2.3.*Transcription factors*

The results of transcriptional profiling that describe grape berry development and ripening showed that several families of transcription factors are involved in such processes (Deluc et al. 2007; Grimplet et al. 2007; Pilati et al. 2007). In our study among the differentially expressed transcription factors the majority belonged to families such as zinc-finger C3HC4, AP2/EFR, MYB, bHLHs, WRKY, Homeobox and NAC (Figure 5).

2.2.3.1.C3HC4-type RING finger

RING finger proteins were modulated in our study by development and water availability. Particularly relevant due to its high representation on the array (more than one hundred entries) was the C3HC4-type RING finger subclass. Interestingly, under RDI the number of differentially expressed *C3HC4-type RING finger* was substantially higher as compared to both FI and NI, suggesting an effect of water status on the modulation of these transcripts. C3HC4-type RING finger proteins were found to play a role on the regulation of Arabidopsis cell cycle (Fleury et al. 2007). Most of them are E3 ubiquitin ligases, therefore very likely to be involved in proteasome-mediated protein degradation (Stone et al. 2005).

2.2.3.2.AP2/EFR

The AP2/ERF superfamily, one of the largest groups of TF in plants, is involved in plants response to drought, salinity, disease resistance, and flowering control (Yamaguchi and Shinozaki 2006). In *V.vinifera* this superfamily comprises more than one hundred genes (Zhuang et al. 2009; Licausi et al. 2010), whose expression was recently investigated in

several grape tissues (Licausi et al. 2010). In our study several *AP2/ERF* were identified, with a clear effect of water status being observed. The majority of these TF were down-regulated during fruit ripening, particularly under FI and NI. Under RDI the number of transcripts positively modulated during maturation was higher as compared to the other conditions, and because this induction was observed at 65DAF it can be suggested that they are involved in the events occurring at *véraison*. Although Licausi et al. (2010) showed that the majority of *AP2/ERF* genes are up-regulated in skin tissues during fruit maturation, when we carefully analyse the list of studied genes we concluded that most of them were not represented in our array.

2.2.3.3.MYB

Several MYBs were differentially expressed during fruit development in our study. MYB proteins are involved in plant development, signal transduction and disease resistance (Jin and Martin 1999; Allan et al. 2008). MYB-transcription factors associated with circadian clock regulation, such as *CC1* and *CIR1/RVE2* will be discussed in section 'other transcription factors'. Particularly relevant to grapes are the MYB proteins that regulate secondary metabolism, in particular the flavonoids pathway. *MYBA1* and *MYBA3* showed a ripening-specific profile in all water status conditions that we studied (see Figure 8). In contrast, *MYBPA1* was negatively modulated by ripening, as expected since it regulates PAs biosynthesis that occurs from fruit set until the beginning of ripening (Bogs et al. 2007; Terrier et al. 2009). The transcriptional regulation of the flavonoids pathway has been largely investigated in *V. vinifera*. VvMybA1 and VvMybA2 are able to induce the *UFGT* transcription, one of the last steps of anthocyanins pathway (Ageorges et al. 2006; Kobayashi et al. 2002; Walker et al. 2007). Moreover, the white phenotype in grapes has been related to the presence of a transposable element, *Gret1*, in the promoter of the *VvMybA1* locus (Kobayashi et al. 2004), showing the relevance of VvMybA1 as a regulator of anthocyanins biosynthesis. In our study, when all the treatments were compared, it was observed that both RDI and NI conditions promoted an early induction of *MybA1*.

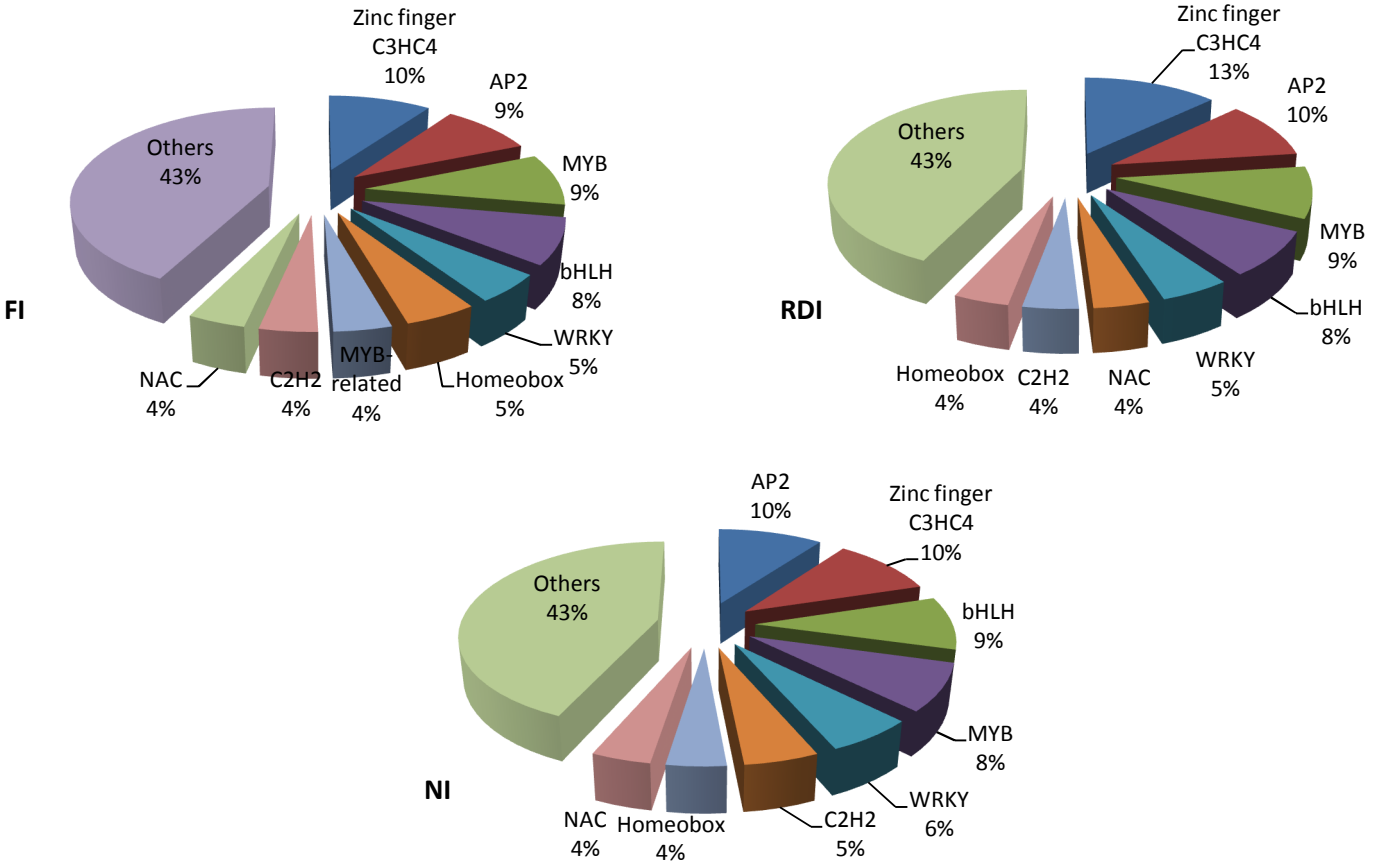

Figure 5 Distribution of transcription factors differentially expressed during berry development and ripening. The major classes of differentially expressed transcription factors are represented in pie charts: FI, RDI and NI conditions.

MybA3 that displayed higher transcripts abundance as compared to *MybA1* (approximately 10-fold higher) also showed to be up-regulated by WD conditions, but in this case at 65DAF *MybA3* was more expressed under NI, followed by RDI and finally FI. However, at 78DAF no difference was observed when comparing NI and FI. The role of *VvMYBA3* is unknown, although Fourrier-Level et al. (2009) suggested that this non-functional VvMybA may compete with the other two functional isogenes (VvMYBA1 and VvMYBA2) during berry pigmentation.

2.2.3.4.bHLH

Several members of Basic Helix-Loop-Helix (bHLH) family were also relevant in the present study. *GLABRA 3* (*GL3*) and *TRANSPARENT TESTA 8* (*TT8*) both members of bHLH family exhibited contrasting expression profiles; *GL3* decreased from 44DAF on (Figure 6A), whereas *TT8* was positively modulated by ripening (Figure 6B). The evidence shows that the activation of different branches of the flavonoids pathway is the consequence of different interactions between bHLH and MYB proteins (Lepiniec et al. 2006). For instance, in *Arabidopsis*, *AtTT8* and *AtGL3* are involved in the control of anthocyanin accumulation in leaves, whereas in seeds *TT8* is necessary for PA accumulation (Nesi et al. 2000; Zhang et al. 2003). So far, in grapevine only two bHLH proteins (VvMYC1 and VvMYCA1) were characterized and potentially involved in both PAs and anthocyanins biosynthesis (Hichri et al. 2010; Matus et al. 2010). *VvMYC1* is the closest homologue of *AtTT8* and in grape berries is expressed throughout development (Hichri et al. 2010), what is in agreement with our results. In our study *VvMYCA1*, an *AtGL3* homologue, was also highly expressed at green berries stages, as also observed by Matus et al. (2010). Further studies will clarify the exact role of these proteins in the regulation of grape berry flavonoids and the role that WD may have on such regulation as we observed that *TT8* was modulated by WD conditions.

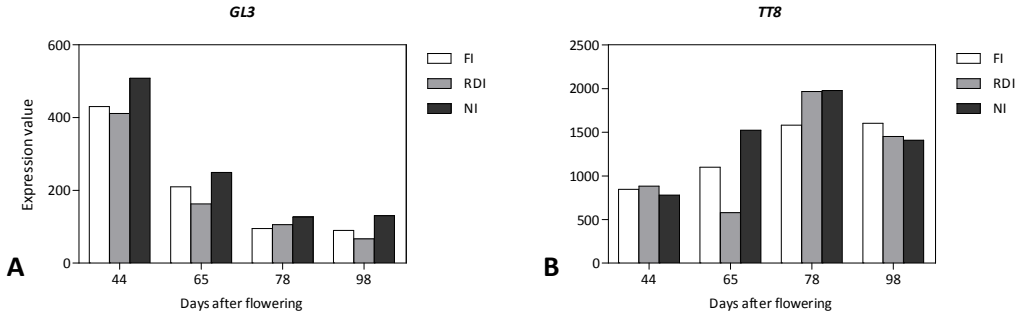

Figure 6 bHLH expression during grape berry development and ripening. Expression profiles of: **A**) *Glabra 3* (*GL3*) and **B**) *TRANSPARENT TESTA 8* (*TT8*) members of bHLH transcription factors during grape ripening and under different irrigation conditions (FI, RDI and NI).

2.2.3.5.Homeobox

The homeodomain–leucine zipper or homeobox (HB) transcription factors have in plants a wide variety of roles (Chan et al. 1998). In the present study we observed that several *HB*s were differentially expressed during fruit ripening. Recently, Gambetta et al. (2010) studied several HBs expression profiles during grape berry ripening and it was shown that several are expressed during fruit ripening. In Arabidopsis HBs have been shown to be involved in ABA responses (Henriksson et al. 2005).

2.2.3.6.NAC

Several *NACs* were also modulated during fruit ripening as previously reported (Pilati et al. 2007). Under RDI conditions the majority of the differentially regulated transcripts were positively modulated, mainly at 65DAF when an accentuated up-regulation was observed. NACs are involved in diverse plant processes such as growth and development but as well in plant responses to biotic and abiotic stresses (Olsen et al. 2005). Recently, Pontin et al. (2010) reported NACs involvement in grapevine leaves in response to UV radiation. Although NACs are expressed in various developmental stages and tissues the details about their role are still largely unknown (Olsen et al. 2005).

2.2.3.7.*WRKY*

Several transcripts of *WRKY* family were differentially expressed during fruit ripening and WD effect was also observed. Most of the transcripts were negatively modulated by

ripening. WRKY participate in numerous cellular processes, such as hormonal signalling, biotic and abiotic stress response (Rushton et al. 2010) and in grape maturation as regulator of sugar signalling network (Gambetta et al. 2010).

2.2.3.8.Other transcription factors

In what concerns MADS-box transcription factors several genes were identified as differentially expressed. Much of the research that has been developed goes into the direction of the role that MADS-box genes have in floral formation/differentiation. Nevertheless, *MADS-box* genes are expressed in wide range of species with fleshy fruits including tomato, banana, apple, grape, and strawberry (Vrebalov et al. 2002; Fei et al. 2004; Malcomber and Kellogg 2005). In grapes several *MADS-box* genes were reported to be related to fruit development (Boss et al. 2001; 2002; Poupin et al. 2007; Díaz-Riquelme et al. 2009). In the present study *SEP1* and *SEP3* (also known as *VvMADS2* and *VvMADS4*, respectively; Boss et al. 2002) were identified as differentially expressed during berry ripening. *SEP1* transcripts decreased from 65DAF onwards. Pilati et al. (2007) also reported the decrease of this gene during fruit maturation. Moreover, under RDI this decrease was earlier observed as compared to both FI and NI. On the other hand, *SEP3* showed to be positively modulated by ripening under RDI and NI, particularly during the transition phase from 44DAF to 65DAF (Figure 7A-B). In agreement with our results, Díaz-Riquelme et al. (2009) showed that *SEP3* expression increased from flowers to mature grapes. In tomato, LeMADS-rin, a *VvSEP1* homologue, was proven to be essential for the ripening process (Vrebalovet al. 2002). The evidence shows that different classes of *SEP* genes have non-redundant and specialized functions in fleshy fruits and are important regulators of the ripening network (Seymour et al. 2011). MADS-box *APETALA1* (*AP1;* Figure 7C) and *AP3* were also differentially expressed during fruit ripening. *AP1* and *AP3* expression decreased from 44DAF onwards and in the case of *AP1* this trend was observed in all studied conditions. Contrarily to our results, *VvAP1* was reported to be absent during grape maturation (Calonje et al. 2004; Díaz-Riquelme et al. 2009).

Transcripts related to light responses (namely CONSTANS-like and circadian clock-related proteins) seemed to be also regulated during fruit ripening and by water status. *CONSTANS* (*CO*), *CONSTANS-like* (*COL*), *flowering locus T protein* (*FT*) transcripts (Sreekantan and Thomas 2006) were shown to be differentially expressed in our study. CO is a central regulator of the network that controls the flowering transition (Tiwari et al. 2010; Valverde 2011). However, the specific biochemical mechanism by which CO proteins regulates the expression of its target genes remains largely unknown (Tiwari et al. 2010). Furthermore, the role of such genes in fruit development and maturation is unknown. We observed that *CC1* (circadian-clock associated 1) was up-regulated from 65DAF onwards in the case of FI and NI berries, whereas under RDI this up-regulation was only observed from 78DAF onwards. It has been suggested that CC1 and COL are also involved in starch mobilization (Lu et al. 2005; Valverde 2011). Also in the present study *CIR1/RVE2* (*CIRCADIAN 1*) was positively modulated during fruit ripening, but no WD effect was observed. Overall, our results confirm that at the transcriptional level circadian clock machinery is active throughout grape berry development and ripening and that water status has an influence on such processes.

We also found *SQUAMOSA Promoter-Binding Protein-Like* (*SPL*) genes modulated by fruit ripening, mostly negatively regulated. However, one of these transcripts, *SPL2*, was only differentially regulated under RDI conditions and showed to be highly expressed at 44DAF, after which it declined (2-fold decreased) to a steady state during fruit ripening. *SPL* genes encode plant specific transcription factors that play important roles e.g. in flower and fruit development, plant architecture and gibberellins signalling (Chen et al. 2010). In tomato one *SPL* gene was proven to be critical for normal fruit ripening (Manning et al. 2006).

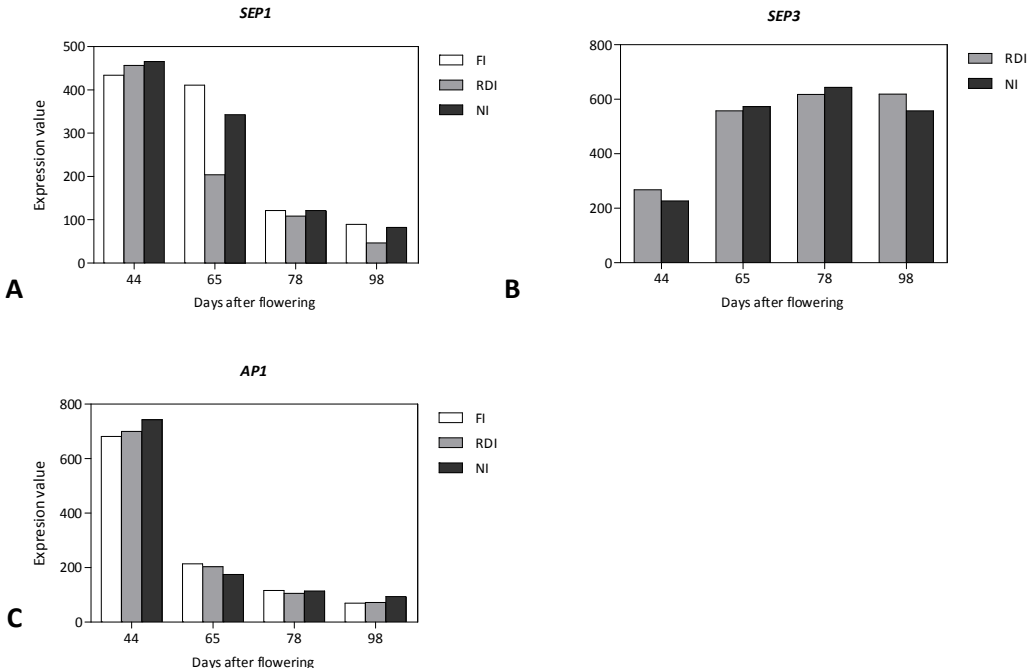

Figure 7 MADS-box transcription factors. Expression profile of MADS-box genes during grape ripening and under different water status conditions (FI, RDI and NI): **A**) *SEPALLATA1* (*SEP1)*; **B**) *SEP3* and **C**) *APETALA1* (*AP1*).

2.2.4.Flavonoids biosynthetic pathway

In general we did not detect a significant increase in anthocyanins content in mature berries induced by WD conditions (Figure 8A). At the transcriptional level the profiles of the major genes involved in grape anthocyanins biosynthesis (Boss et al. 1996) confirmed this observation. However, we observed that under RDI anthocyanins start to accumulate earlier than FI and NI berries ($p<0.05$). At the general branch of phenylpropanoids it was observed an overall induction of *phenylalanine ammonia-lyase* (*PAL*), *cinnamate 4-hydroxylase* (*C4H*) and *4-coumarate-CoA ligase* (*4CL*), suggesting that 4-coumaryl-CoA is being formed to feed the downstream pathways. The genes coding *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*) were as well induced from *véraison* onwards, confirming previous data (Boss et al 1996). Also the genes coding for the enzymes that have a direct action in the anthocyanins biosynthesis such as *leucoanthocyanidin dioxygenase* (*LDOX*) and *UDP-glucose:flavonoid 3-O-*

glucosyltransferase (*UFGT*) were as well induced. A strong correlation (r>0.97) between *UFGT* and *MYBA* transcriptional profile was observed in all conditions (Figure 8B-D). Robust correlations (r>0.80-0.97) were observed between *UFGT* expression profile and anthocyanins content (mg/berry) under both FI and RDI conditions. Whereas NI berries showed weak correlation (r>0.56). At this point no explanation could be ruled out for this somehow conflicting result observed in NI berries, except the observation regarding *MYBA3* expression pattern. It might be that the mild WD induced in RDI vines is the optimal trigger of the transcripts underlying anthocyanin synthesis. *Flavonol synthase* (*FLS*) gene which is required for flavonol biosynthesis was positively modulated during fruit ripening in all conditions but under WD the induction was much more expressive. Under FI, *FLS* was up-regulated from 78DAF onwards, whereas under RDI and NI this up-regulation was observed already at 65DAF. Under NI at 78 and 98DAF the highest expression was observed, displaying at least 15-fold induction as compared to FI berries. *Anthocyanidin reductase* (*ANR*) showed to be highly expressed at 44DAF and then steady decreased during fruit ripening, in accordance with its specific involvement in proanthocyanidins (PAs) biosynthesis (Terrier et al. 2009). In grape berries both Multidrug and Toxic compound Extrusion (MATE) proteins (Gomez et al. 2009) and ATP-binding cassette (ABC) transporters (Francisco et al. unpublished results) are involved in the vacuolar sequestration of anthocyanins. Moreover, considerable evidence points that GSTs are involved in such process (Alfenito et al. 1998; Kitamura et al. 2004), whether as a *ligandin* or through substrate glutathionation. Several MATE and ABC transporters were also identified in our study as differentially expressed during fruit ripening, some were positively whereas others negatively modulated during fruit ripening. We recently collected evidence that an ABC protein (VvABCC1) is able to transport *in vitro* glucosylated anthocyanins (Francisco et al. unpublished results; see Chapter IV), but we believe that this gene is not represented in our array. From the several genes coding for *glutathione S-transferases* (*GSTs*), one *GSTF12* (VVTU13534_s_at) showed a strong correlation either with *UFGT* and *MYB* expression

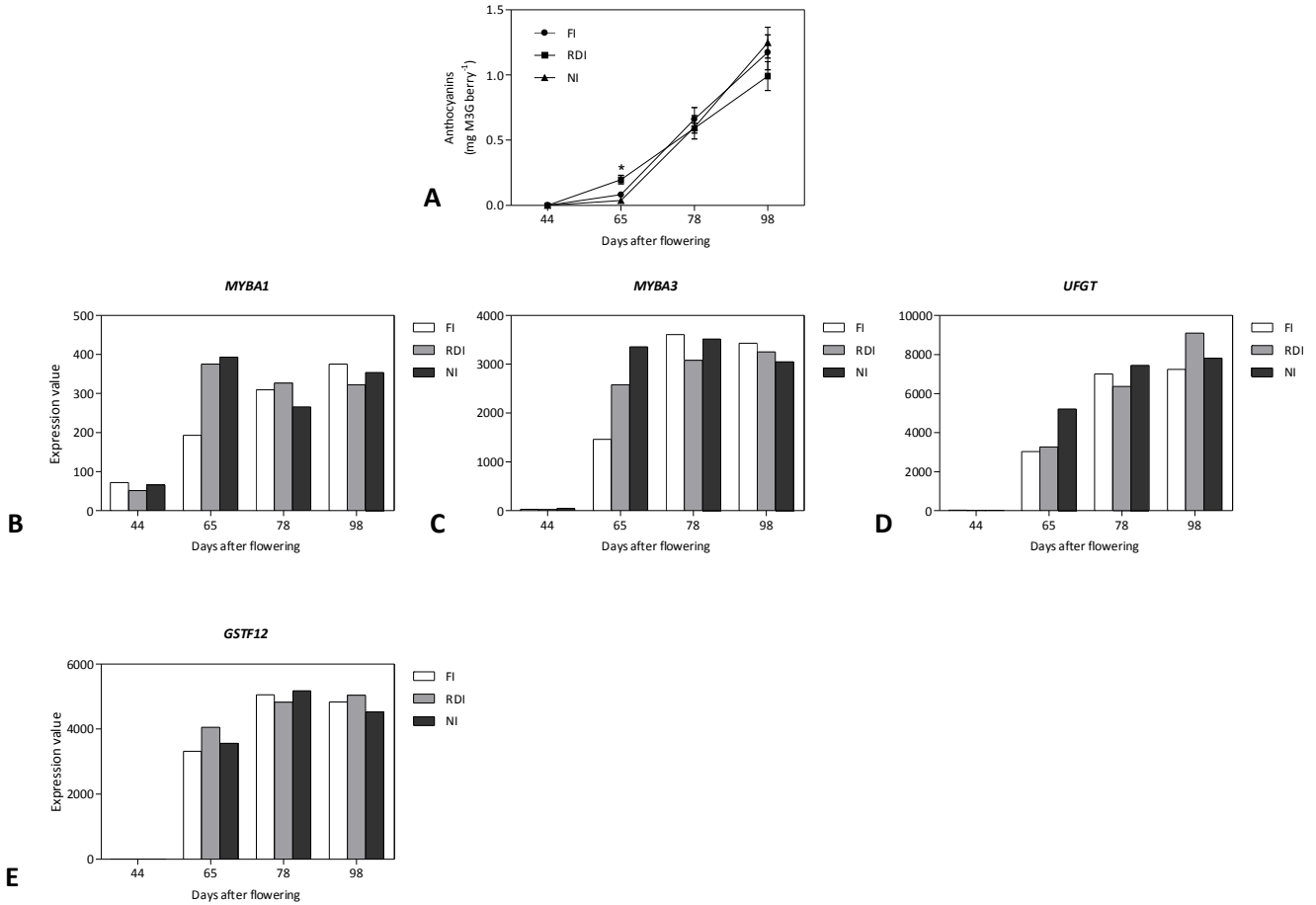

Figure 8 The influence of water deficit conditions in grape anthocyanins metabolism. A) Profile of anthocyanins accumulation in grape berries during ripening under the effect of FI, RDI and NI. The expression profile of transcripts that are directly involved in anthocyanins metabolism **B**)*MYBA1*; **C**)*MYBA3*; **D**) *UDP-glucose:flavonoid 3-O-glucosyltransferase* (*UFGT*) and **E**) *glutathione S-transferase F12* (*GSTF12*); * indicate significance differences between irrigation treatments ($p<0.05$); bars represent ± SE.

patterns (r>0.97). Its expression pattern was conserved in all water status conditions (Figure 8E). Ageorges et al. (2006) also observed the co-expression of *GST* with several genes involved in anthocyanins biosynthesis. GSTF12 is one of the best functionally characterized GSTs in plants (Dixon and Edwards 2010). In Arabidopsis these GSTs are involved in both anthocyanins and PA vacuolar sequestration (Kitamura et al. 2004).

2.2.5.Sugars metabolism

Sugars produced in leaves are transported to berries through the phloem. At the onset of *véraison* the unloading of sugars shifts from symplastic (via plasmodesmata) to apoplastic (Zhang et al. 2006). Hexoses (mostly glucose and fructose) begin to accumulate in the lag phase and continue afterwards.

In this study we determined that in pericarp tissues glucose and fructose content was similar at harvest in all water status conditions (Figure 9A). Moreover sucrose content remained relatively low as compared to hexoses. However, the onset of hexoses accumulation was significantly altered by WD. It was observed that both RDI and NI berries start to accumulate earlier hexoses, with a sharper increase observed under RDI conditions. The metabolism of sugars accumulation is most relevant in pulp tissues as the main sugar storage tissue in grape berries. Still we observed that in skin tissues several sugar-related transcripts were regulated in a developmental/ripening manner. In fact, a similar pattern of hexose accumulation is observed in the skin and in the pulp during berry maturation (Davies and Robinson 1996), so some overlapping results may be expected, although sugar concentrations in skin are generally lower than those present in the flesh tissue (Davies and Böttcher 2009).

Vacuolar invertases (*GIN1* and *GIN2*) were modulated by ripening. *GIN1* was progressively down-regulated during ripening in all water status conditions. However, at 44DAF the relative transcript abundance was lower under NI conditions compared to both FI and RDI. *GIN2* showed a transient up-regulation at 65DAF that was most expressive under FI and NI conditions (Figure 9B-C). *GIN* expression is high in early stages of berry development but declines when hexoses start to accumulate,

concomitantly with the shift of sugars unloading from symplastic to apoplastic (Zhang et al. 2006). We also observed that under RDI conditions a *neutral/alkaline invertase* (VVTU222_at) was differentially expressed and its decrease was observed at the onset of *véraison* (65DAF). Nonis et al. (2008) identified 9 neutral/alkaline invertases in *V.vinifera* (in the 8X genome assembly) where the same trends of expression in mesocarp and exocarp tissues were observed, though the transcripts were more expressed at the skin, but still their precise role in sugar metabolism remains unknown. We observed that *sucrose synthase* (*SuSy*) declined from 44 to 65DAF, after which a steadily expression was observed under RDI conditions whereas under NI an up-regulation was observed at 78 DAF (Figure 9D). SuSys covert sucrose into UDP-glucose and fructose and it has been suggested that it can participate in cell wall metabolism during grape ripening (Deluc et al. 2007) since during this period cell wall invertases appear to play the main role in the cleavage of sucrose (Zhang et al. 2006). We also observed that *sucrose-phosphate synthase* (*SPS*) genes were positively modulated by ripening and the observed trend was mirrored in all water status conditions (Figure 9E-G). This trend supports the model where sucrose is re-synthesized following its unloading. This mechanisms has been proposed to favour the unloading process and storage of sugars during fruit ripening (Nguyen-Quoc and Foyer 2001; Agasse et al. 2009). Sugar transporters were also identified in the present study. *Hexose transporter* (*HT2*) was transiently up-regulated at 65DAF in all conditions, after which its decrease was observed (Figure 10A). *VvHT2* profile is most specifically associated with *véraison* stage (Terrier et al. 2005; Afoufa-Bastien et al. 2010). In grape berries, the available data show that *VvHT1*, *VvHT2* and *VvHT3* are highly expressed throughout all developmental stages, and it has been speculated that monosaccharide import into berries during ripening is mainly due to VvHT2 but mostly to VvHT3 action, although their sugar transport activity has not yet been established (Agasse et al. 2009). A *sucrose transporter* (*SUC2*) was also differentially regulated during fruit ripening being negatively modulated in all conditions

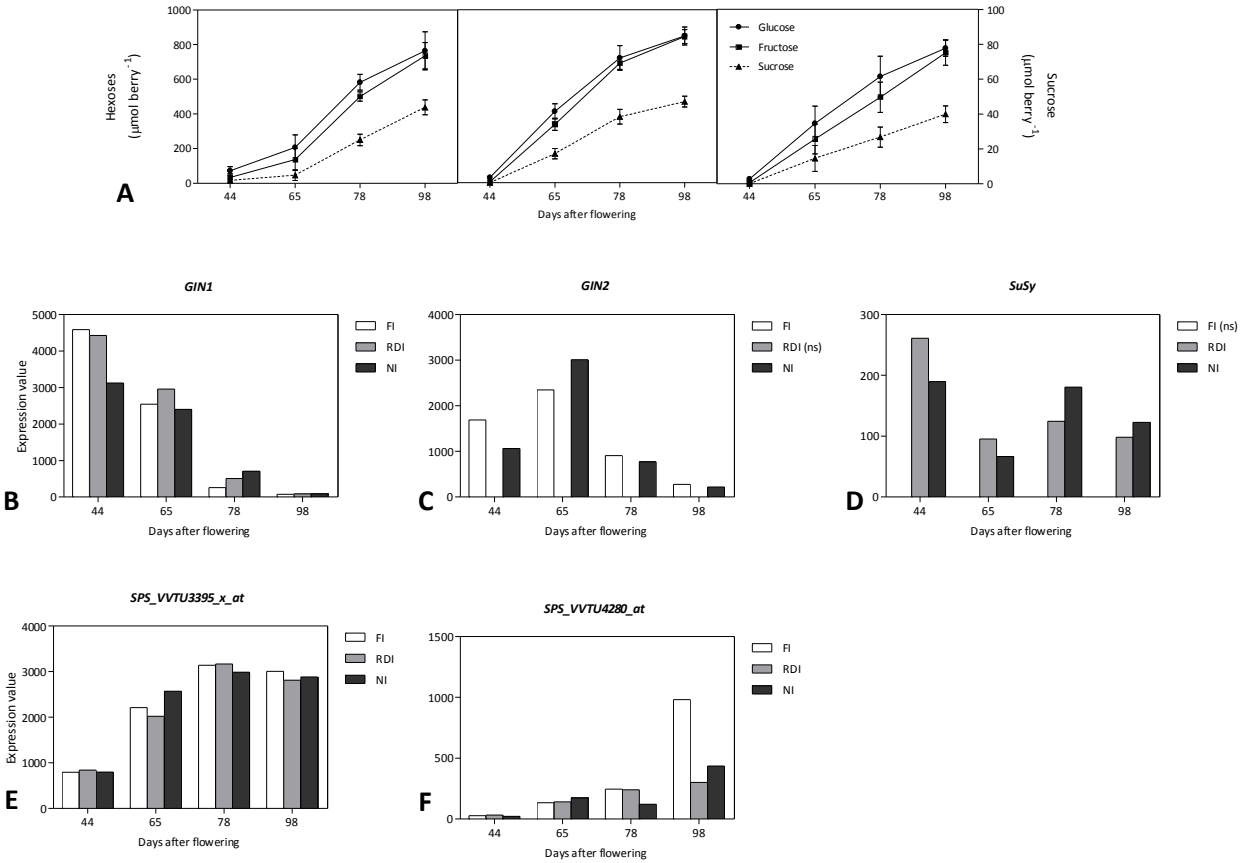

Figure 9 Sugars metabolism during grape ripening. A) Sugars accumulation profile during berry ripening under FI (Left panel), RDI (central panel) and NI (right panel). Expression profile of sugars related genes differentially expressed during ripening: **B**) *Vacuolar invertase I* (*GIN1*); **C**) *GIN2;* **D**) *sucrose synthase* (*SuSy*) and **E-F**) *sucrose-phosphate synthase* (*SPS*) profiles. Bars represent ± SE.

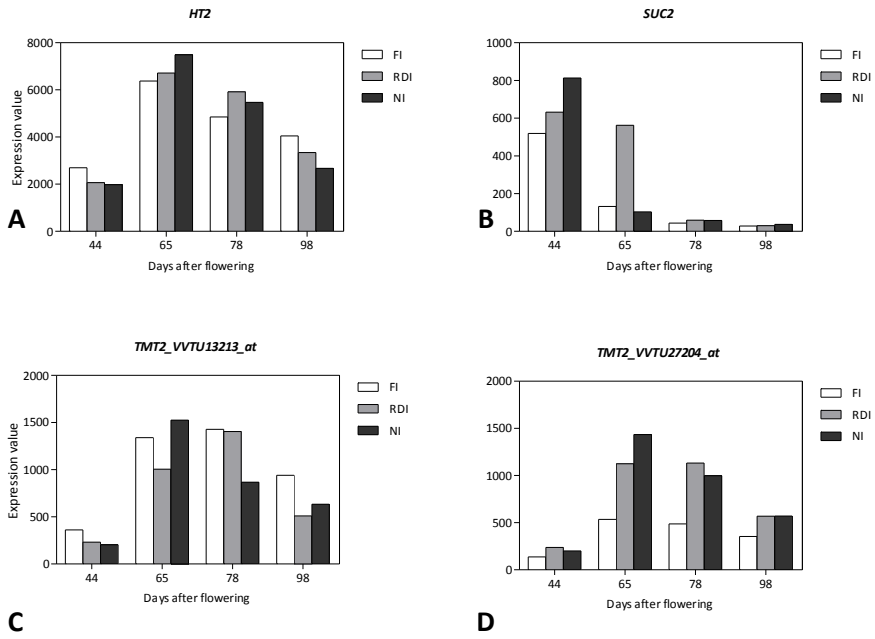

Figure 10 Grape skin sugar transporters. Differentially expressed transcripts during fruit ripening and under different water status conditions (FI, RDI, NI): **A**) *hexose transporter 2* (*HT2*); **B**) *sucrose transporter 2* (*SUC2*); **C-D**) *tonoplastic monosaccharide transporter2* (*TMT2*). 'VVTU' represents probeset ID.

however this pattern under RDI was observed only from 65DAF onwards, whereas under FI and NI it was already detected at 65DAF (Figure 10B).This transcript showed homology with *VvSUC27* which previously has been shown to decline over the course of berry ripening (Davies et al. 1999). *TMT2* (*tonoplastic monosaccharide transporter2*) were modulated by water status (Figure 10C-E), particularly *TMT2* isogene (VVTU27204_at) that under WD condition its induction was observed from 65DAF onwards. Although their transport activity is still not proven (Afoufa-Bastien et al. 2010), it has been suggested that they could be involved in vacuolar hexose accumulation. In Arabidopsis *TMT2* expression is induced by drought, salt, and cold treatments and by sugar (Wormit et al. 2006). Several transcripts described as *ERD6-like* transporters were differentially expressed in our study. The majority was negatively modulated by ripening. Two transcripts were the most relative highly expressed: *ERD6-like 6* and *ERD6-*

like 7. Again we observed that WD did not impose any expression profile alteration; *ERD6-like 6* was up-regulated at 65DAF, decreasing thereafter, whereas *ERD6-like 7* decreased from 44DAF onwards. These putative sugar transporters were named after the identification of *AtERD6* (*early-responsive to dehydration*) gene, which is coding for a putative sugar transporter (Büttner 2007). Recently, Afoufa-Bastien et al. (2010) identified in the *V.vinifera* translated genome 22 *ERD6-like* transporters. In *A.thaliana* a member of this family *AtERD6-1* was reported to function in the efflux of hexoses from the vacuole to the cytoplasm as a mechanism to regulate sugar remobilization and osmotic pressure under abiotic stress conditions (Yamada et al. 2010).

We also observed that starch metabolism, mostly the genes related to starch catabolism, is active in berry skins during grape ripening agreeing with the results obtained in pericarp tissues by Deluc et al. (2007). Under FI the most highly expressed transcripts were two β-*amylases* that sharply increased from 44DAF (VVTU15830_s_at) and 65DAF (VVTU37021_s_at) onwards. Under RDI, we observed that also β-*amylases* were modulated. One (VVTU15830_s_at) showed the same trend as the one described to FI, while β-*amylase* (VVTU12925_at) showed a transient 17-fold increase at 65DAF. Under NI the number of differentially regulated transcripts related to starch metabolism was reduced but still the most expressed transcript was also a β-*amylase* (VVTU15830_s_at) that showed exactly the same trend as previously described in both FI and RDI. Deluc et al. (2007) quantified starch concentration in grape berries, and it was observed that it significantly declined during Phase III of berry development. Interestingly, we observed (predominantly after 65DAF) the activation of circadian clock genes (namely *CC1*) that have been described as putative regulators of starch degrading enzymes (Lu et al. 2005). In strawberry starch degradation predominates during fruit growth and development, and could contribute up to 3% of the sugar accumulated in ripe fruit (Souleyre et al. 2004). Overall, this agrees with the pattern that we observed where starch reserves can contribute to sugar content in ripe berries.

Trehalose metabolism was also modulated in the present study. We observed that *trehalose 6-phosphate synthase* (*TPS*) and *trehalose-6-phosphate phosphatase* (*TPP*)

were differentially expressed; *TPS* increased during fruit ripening, this increase being more pronounced under FI, particularly at 78 and 98DAF(Figure 11A). On the other hand, *TPP* expression profile was characterised by a transient increase at 65DAF under RDI, while under NI this pattern was observed at 44DAF. *TPP* relative expression under FI was lower as compared to WD conditions (Figure 11B). Interestingly, it seems that trehalose-6 phosphate (TP6) synthesis, the precursor of trehalose, is favoured during the later stages of fruit maturation, whereas trehalose synthesis is only induced in precise moments of berry development. The evidence shows that T6P is a signalling molecule that putatively coordinates metabolism in response to carbon availability and stress, regulating CW and starch metabolism for example (Paul et al. 2008, 2010). Still the function of trehalose in plants must be established (Paul et al. 2008). Grimplet et al. (2009) determined that trehalose was predominantly found in skin and seed tissues of mature berries. The quantification of T6P has proven difficult due to its low abundance in plant tissues, but recently a method has been developed (Delatte et al. 2011) that could be used in future research studies to uncover TP6 role during grape ripening.

2.2.6.Cell wall metabolism

2.2.6.1.Cell wall disassembly

Plant cell walls consist of cellulose microfibrils and matrix substances (pectins, hemicelluloses, proteins and phenolics). The structural and biochemical modifications that skin and pulp cells undergo during development that allow cell expansion and softening have been scrutinized in the past years (Nunan et al. 1998, 2001; Robinson and Davies 2000; Deytieux-Belleau et al. 2008; Vicens et al. 2009; Goulão et al. *in press*). Cell expansion requires primary cell wall loosen and also the incorporation of new material. Fruit softening is associated with cell wall disassembly, where both pectic and hemicellulosic compounds undergo solubilization and depolymerization (Rose et al. 1999). Within the pectin-related metabolism several *pectinesterase* (*PME*) and *pectate lyase* (*PL*) transcripts were identified as differentially expressed, although a pattern regarding the ripening events could not be identified. Deytieux-Belleau et al. (2008)

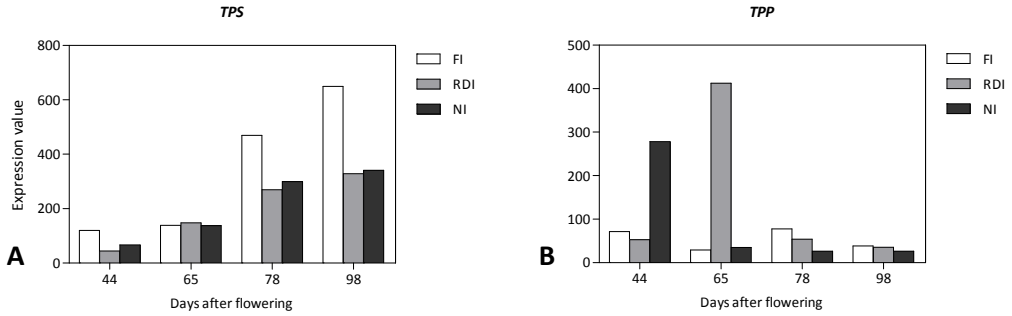

Figure 11 Grape skin trehalose metabolism. The expression profile of **A**) *trehalose 6-phosphate synthase* (*TPS*) and **B**) *trehalose-6-phosphate phosphatase* (*TPP*) during grape berry ripening and under different water status conditions (FI, RDI and NI).

showed that both *VvPME1* mRNA and PME activity were detected in berry skin prior to *véraison*, suggesting that PME role is not restricted to the ripening process. In fact, several other reports showed that *PME*s are present at different times during berry development (Terrier et al. 2005; Deluc et al. 2007). Regarding *polygalacturonase* (*PG*), we observed an up-regulation of several PGs under NI as compared to FI and RDI, particularly post-*véraison*. Although the data in the literature is not consensual in what concerns PG activity during grape ripening (Cabanne et al. 2001; Nunan et al. 2001; Deytieux-Belleau et al. 2008), the accumulation of *PG* transcripts seems to be correlated with softening and increased during anthocyanins accumulation (Deytieux-Belleau et al. 2008). We also observed that under FI and RDI, transcripts codifying PG inhibitor proteins were highly expressed at 65DAF, being its relative expression higher under RDI conditions. These are ubiquitous plant cell wall proteins that are directed against fungal PGs (De Lorenzo and Ferrari 2002). However, in our study their expression profile is apparently more related with the ripening programme than plant defence. Deluc et al. (2009) also identified a PG inhibitor that could regulate PG activity (Tornielli et al. *in press*) but further research is needed to support this speculation. Regarding *xyloglucan endotransglucosylase* (*XTH*), the higher number of differentially expressed genes was

observed under RDI, followed by NI and FI. It was observed that these genes were expressed during all sampling dates. However, the most dramatic alterations as a result of WD were observed at 65DAF. The highest express transcript, xyloglucan endotransglucosylase/hydrolase 32 (VVTU12717_s_at), displayed a 174-fold increase in expression between the transition from 44 to 65DAF in FI berries. Under WD this increase was even more expressive with 272 and 400-fold change under RDI and NI, respectively. XTH gene product was suggested to cleave cellulose–xyloglucan network inducing grape berry softening at *véraison* (Ishimaru and Kobayashi 2002). We also detected two β-*expansins* (*EXPL2* and *EXPB4*), which were highly expressed; *EXPL2* decreased as a whole during fruit ripening under FI, whereas under RDI almost 10-fold induction was observed from 44 to 65DAF, after which this transcript decreased. Under NI, *EXPL2* showed to be more expressed at 44DAF following thereafter a steady decrease. *EXPB4* was positively modulated by ripening, with the highest relative expression observed under FI, particularly at 78 and 98DAF. Two other expansins, in this case α-type, were also identified. *EXPA8* was relatively more expressed than *EXPA15*, but the same expression profile was observed. It significantly increased at 65DAF, being the lowest expression observed under RDI. At 98DAF under NI conditions both transcripts were repressed as compared to FI. Overall, this suggests that expansins are modulated by WD during fruit ripening. Expansins were proposed as a class of proteins that act as wall-loosening agents (Cosgrove 2000) and are abundantly expressed in softening fruits, although the mechanisms by which expansins induced fruit softening still remain obscure (Ishimaru et al. 2007). It has been proposed that they might contribute to cell-wall degradation by increasing the accessibility of other cell-wall modifying proteins, namely PGs (Rose and Bennett 1999). Several other transcripts related to cell wall metabolism were also identified in the present study as differentially regulated during fruit ripening, such as *hydroxyproline-rich glycoproteins* (*HRGPs*), *cellulose synthase*, *cellulase*, *COBRA* or *FLA1* and *FLA2* (*fasciclin arabinogalactan-protein*).

2.2.6.2.*Lignin biosynthesis*

Cinnamoyl-CoA reductase (*CCR*), *cinnamyl alcohol dehydrogenase* (*CAD*), f*erulate-5-hydroxylase* (*F5H*) and *laccase*, all involved in lignin biosynthesis, were also identified as differentially expressed during fruit ripening. Water status had a pronounced effect on *CCR* and *CAD* expression profiles. Under FI one *CCR* (VVTU3517_at) increased until 78DAF, after which its down-regulation was observed, whereas other *CCR* gene (VVTU13147_s_at) was negatively modulated from 44DAF onwards. *CAD* was mostly increasingly up-regulated during fruit ripening. Under RDI, the majority of *CCR* genes suffered a significant down-regulation at 65DAF, after which a modest increase was observed until later stages of ripening, with the exception of one *CCR* (VVTU3517_at) that was positively modulated by ripening, but relatively less expressed as compared to FI. Also *CAD* and *F5H* were negatively modulated by ripening under this condition. Regarding NI conditions, a positive effect of ripening was observed either in what concerns *CCR* or *CAD* transcripts. In fruits, lignin is associated to vascular tissues (Sarkar et al. 2009), and it was suggested that vascular development including lignification is an integral process in strawberry development and ripening transcriptional program (Aharoni et al. 2002).

In general, we observed that most of the transcripts associated with cell wall metabolic functions are altered by WD, although the complexity of cell wall metabolism and the mechanisms that control fruit softening forbids us to fully apprehend the observed alterations. Several attempts have been made to characterize cell wall modifications during ripening (Giovannoni 2001) but there is still a lack of a detailed understanding about cell wall degradation in the process of fruit softening (Sarkar et al. 2009).

2.2.7.Aquaporins

Aquaporins were also detected as developmentally regulated. Several PIPs (plasma membrane intrinsic proteins) were differentially regulated in our study (Figure 12A-C). *PIP2;2* (ex PIP2B accordingly to Johanson et al. 2001; in our study identified as VVTU29911_at) was up-regulated during fruit ripening in particular under WD, the

highest relative expression being observed under RDI over the course of fruit ripening. However, contrasting results have been reported. Both Fouquet et al. (2008) and Choat et al. (2009) showed that in whole berries *PIP2;2* expression decreases after *véraison*. Further evidence needs to be collected to confirm that in skin tissues *PIP2;2* has a different expression pattern. *PIP2;4* showed under FI a peak of expression at 65DAF after which its expression stabilized; under NI the same peak was observed but it was followed by a steady decrease; finally under RDI its expression was lower as compared to both FI and NI but its peak was observed at 78DAF, after which it decreased. *PIP2;7* (PIP3) showed to be negatively modulated by ripening under RDI and NI, however under FI its decrease at 65DAF was followed by an up-regulation until later stages of berry ripening. Also TIPs (tonoplast intrinsic proteins) showed to be differentially regulated (Figure 12D-F). *TIP1;1* (γTIP; VVTU18118_s_at) and *TIP1;3* (VVTU7389_s_at) were highly expressed, showing a peak of expression at 65DAF, after which a decline was observed. While in the case of *TIP1;1* no WD effect was observed, *TIP1;3* was significantly more expressed under NI, followed by RDI conditions. *TIP2;1* (δTIP) showed under FI and RDI a decrease, in the case of FI after 65DAF whereas under RDI already at 65DAF. Under NI this transcript showed a relatively lower expression. Fouquet et al. (2008) showed that *TIP2;1* is highly expressed in xylem cells before *véraison*, after which a strong decline is observed, concomitant with the shift that occurs from xylem to phloem water uptake (Greenspan et al. 1994). Overall, the major trend observed was a negative modulation of TIPs by ripening. Finally, a *NIP5;1* (*nodulin 26-like intrinsic protein*) was negatively modulated by ripening in all water status conditions (Figure 12G). Although, as far as we know, there is no information about NIPs role in grapevines, its involvement in grape

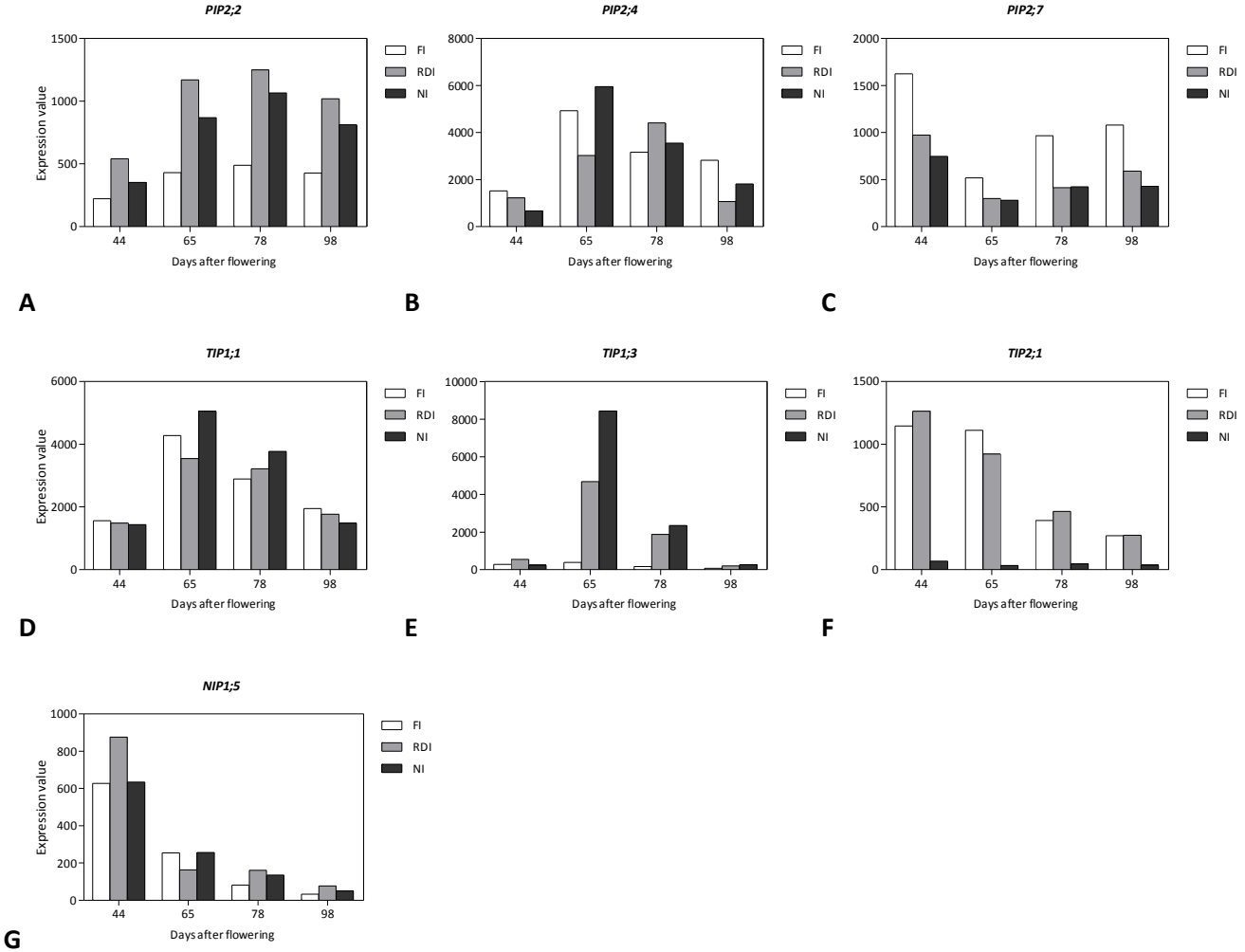

Figure 12 Aquaporins expression during fruit ripening and WD effects. Aquaporins modulation during grape ripening of FI, RDI and NI berries: **A**) *PIP2;2*; **B**)*PIP2;4*; **C**)*PIP2;7*; **D**)*TIP1;1*; **E**)*TIP1;3*; **F**)*TIP2;*1 and **G**)*NIP1;5*.

berries apparently is more related to the initial stages of berry development. NIPs functions are still poorly characterized. In Arabidopsis, apart from water transport its involvement in nutrient uptake was proposed since AtNIP5;1 was proven to be involved in boron transport (Takano et al. 2006).

In general, we observed that different aquaporins are differentially expressed during fruit ripening, and that WD conditions also modulate their expression profile. It is important to stress that it cannot be excluded that aquaporins might transport other molecules apart from water (Tyerman et al. *in press*). As Choat et al. (2009) suggested, aquaporin expression may not significantly impact hydraulic resistance of the whole berry but may contribute to the ripening process in other ways. The ripening process is associated with large increases in sugar transport and accumulation, changes in cell wall metabolism, and changes in turgor, all of which are certainly impacted by aquaporins and their modulation of membrane water permeability (Choat et al. 2009).

2.2.8.Stress metabolism

During fruit development and ripening several components of plant 'stress metabolism' are activated. Three ELIP1 (Early light-inducible protein) genes were differentially expressed; however, one ELIP1 (VVTU26995_x_at) showed to be highly expressed under FI conditions as compared to WD berries, 4-fold induced in all analysed stages. In tomato ELIP proteins play a role in the chloroplast-to-chromoplast transition process during ripening (Bruno and Wetzel 2004). Since grape berries possibly contain intact and functional plastids during fruit ripening (Diakou and Carde 2001), these proteins may be relevant during grape berry ripening. Also, we observed that the one helix protein (OHP) and one stress enhanced protein 1 (SEP1) gene products, all related to high light stress, decrease in all conditions during fruit ripening. *Late embryogenesis abundant protein* (*LEA*) transcripts showed to be regulated at the transcriptional level. Under FI, we observed a down-regulation from 44DAF onwards; on the other hand RDI and NI

showed increased *LEA* expression at later stages of berry ripening. *Dehydrin 1b*[4] was positively modulated by ripening, but WD conditions had a pronounced effect on this up-regulation, particularly from 65DAF onwards. LEA proteins accumulation is tightly correlated with acquisition of desiccation tolerance, mostly to their capacity to stabilize other proteins and membranes during dehydration process (Hand et al. 2011). *RD22* transcripts where in all conditions more expressed at 44 and 65DAF, after which a general decrease was observed. RD22, a dehydration responsive protein, was reported to be positively regulated at the transcriptional level by ripening (Deluc et al. 2007) and in mature berries to decrease as a result of WD conditions (Grimplet et al. 2007). In our case, however, no WD effect was observed. Transcripts related to oxidative stress, namely *peroxidase*, *glutaredoxin*, *polyphenol oxidase*, *ascorbate peroxidase* and *catalase* were also differentially expressed during fruit ripening. Under RDI we observed higher number of differentially regulated genes related to oxidative stress than in FI and NI. We also observed a complex pattern where the same family of transcripts appear positively or negatively modulated by ripening, suggesting that in the different stages of berry ripening different classes of antioxidant transcripts are operating. Pathogenesis-related proteins are the most abundant proteins in grape berries, in particular thaumatin-like proteins (TLPs) and class IV chitinases (Chi4), as reported by several studies (Salzman et al. 1998; Pococok et al. 2000; Robinson et al. 1997; Tattersall et al. 1997). Not surprisingly their transcripts were highly expressed mainly from 65DAF onwards, no water status effect was detected. *ER6 protein universal stress protein* transcripts were on the other hand only differentially expressed under WD conditions. The same trend was observed in both conditions, *ER6* was highly expressed at 44DAF, after which a it decreased and then a steady expression was observed at later stages of berry ripening. Under FI *ER6* was steadily expressed in all studied sampling dates. This gene was shown to be ethylene-regulated during tomato ripening (Zegzouti et al. 1999). *PR-1* transcripts showed to be up-regulated at 44DAF, after which a decrease was observed, as previously reported (Deluc et al. 2007). We also observed an effect of WD at 44DAF

[4] Dehydrins belong to group 2 of LEA proteins (Battaglia et al. 2008).

since the relative expression of these transcripts was higher in NI followed by RDI as compared to FI. Many defence-related proteins, such as PR-1, are inducible by the signalling compounds such as salicylic acid (SA). *SEN1* (*DARK INDUCIBLE 1*) transcripts showed a transient 50-fold induction at 65DAF under RDI conditions. In Arabidopsis this gene is regulated predominantly via the SA and jasmonic acid signalling pathways (Schenk et al. 2005). *ORG1* (*OBP3-RESPONSIVE GENE 1*) transcript was modulated during fruit ripening. Under FI, a transient down-regulation was observed at 65DAF. On the other hand, an over-expression was observed at 44 and 65DAF under NI and RDI respectively, after which the transcript decreased to the same relative expression as observed under FI. OBP3 is a DOF (DNA binding with one finger) protein, which plays an important role in plant growth and development (Kang and Singh 2000). ORG1, an OBP3 responsive gene encodes a protein kinase and has been found to be induced in response to SA (Kang et al. 2003). SA is a signal in plants response to stress and although no function has been attributed to SA during fruit ripening (Davies and Böttcher 2009) it was interesting to observe that transcripts such as PR-1, SEN1 or ORG1, described in other species as SA-responsive, were all induced in early berry stages and decreased around *véraison*. Several other transcripts involved in both biotic and abiotic stress that were differentially regulated during fruit ripening are presented in (Additional file 1).

3. Conclusions

Grape berry skin transcriptome was differentially affected by fruit ripening and by water status conditions. However, we observed at the transcriptional level a conservation of some of the most relevant processes during fruit ripening, confirming the complexity of the transcriptional regulatory hierarchies that control ripening events. Moreover, mRNA profiles of RDI berries were those that in general presented singular expression profiles when compared to both FI and NI conditions, corroborating that the imposed irrigation strategy is able to alter berry metabolism, with sugar and anthocyanins accumulation being anticipated under a mild water deficits as observed under RDI conditions. Some of most relevant processes that occur during fruit ripening were reviewed and the effect of

water deficits assessed. The transcriptional regulation of anthocyanins biosynthesis agrees with anthocyanin accumulation except at 65DAF under NI conditions, what raises some further questions. Concerning the hormonal regulation, the observed alterations suggest that ABA metabolism may be internally regulated by a dynamic balance between biosynthesis and catabolism events. Moreover, ethylene, auxins and brassinosteroids and cytokinins were also under WD modulation. The activation of several components of signalling, namely peptide or circadian clock signalling metabolism was observed in all water status conditions, being apparently more related with ripening events themselves than with berry adjustments to adverse conditions. We also observed that sugar metabolism follows a similar trend as described to pulp tissues, and that trehalose metabolism was modulated by WD conditions. T6P, the precursor of trehalose may be part of the sugar metabolism regulation. The high number of transcription factors differentially regulated corroborates the fact that ripening is under a tight transcriptional regulation. Actually, the expression profile of several bHLH, MADS-box, Squamosa-like strongly suggests a role during fruit ripening. In conclusion, we observed that WD impacted mRNA expression patterns, but apparently these alterations are more subtle than has been previously reported.

4. Materials and Methods

4.1. Plant Material - Grape irrigation treatments and berry sampling

During the summer season of 2006 irrigation treatments were imposed to five year-old *Vitis vinifera* var. Aragonez plants (grafted on 1103 Paulsen rootstock), trained on a bilateral Royat Cordon system. Plants were drip irrigated at both sides of the row with 2 Lh^{-1} drippers supplying either 100% Etc (Full irrigation, FI) or 40% Etc (Regulated-deficit irrigation, RDI). Irrigation in RDI started from beginning of June until 90% of véraison was attained, whereas FI vines started to be irrigated from end May until beginning of September. Non-irrigated (NI) but rain-fed grapevines were also studied. Leaf ψ_{pd} were measured with a Scholander-type pressure chamber (Model 1000; PMS Instrument Company, Corvallis OR, USA) from the beginning of berry development until harvest.

Berries were collected from the pea size until full maturation was attained, corresponding the sampling dates to 44, 65, 78, 98 days after flowering (DAF). Véraison was considered when 50% of the berries were coloured. At the reported days four biological replicates per treatment (composed by 15 clusters each) were harvested, at midday in both sides of the vines, immediately immersed in liquid nitrogen and stored at -80ºC until use.

4.2. Metabolite analysis

Grape berry sugars and anthocyanins were measured as described (Francisco et al. under submission; see Chapter III).

4.3. RNA isolation and GeneChip hybridization

Total RNA was extracted from skin berries (5-15 berries) using the method described by Reid et al. (2006). Briefly, skins were ground in liquid nitrogen into a fine powder. The extraction buffer, pre-warm at 65°C (300 mM Tris HCl, pH8.0, 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVPP, 0.05% spermidine trihydrochloride and 2% β-mercaptoethanol) was added (15 mL/g FW) to the powder and incubate for 10 min at 65°C under a vigorous shaken. An equal volume of chloroform: isoamyl alcohol (24:1) was added and plant material was centrifuged at 3,500 × *g* for 15 min at 4°C. The aqueous layer was transferred to a new tube, and chloroform: isoamyl alcohol (24:1) was again added, and samples were centrifuged at 30,000 × *g* for 20 min at 4°C. To the collected supernatant 0.1 vol 3 M NaOAc (pH 5.2) and 0.6 vol isopropanol was added, mixed, and incubated at -80°C for 30 min. Nucleic acid were collected by centrifugation at 3,500 × *g* for 30 min at 4°C. The pellet was dissolved in 1 ml TE (pH 7.5) and transferred to a microcentrifuge tube. RNA was precipitated (0.3 vol of 8 M LiCl) and stored overnight at 4°C. RNA was pelleted by centrifugation (20,000 × g for 30 min at 4°C), washed with cold 70% ethanol, air dried and dissolved in 100 μL DEPC-treated water. Total RNA was purified using RNeasy Mini kit (Qiagen) with DNase I digestion included according to standard protocols. Samples were analysed at the Genomics Unit of the Spanish National Centre for Biotechnology (CNB-CSIC, Madrid). RNA integrity analyses were performed with an Agilent's Bioanalyzer 2100. Probe synthesis, microarrays hybridization, washing, staining

and scanning with the GeneChip™ Scanner 3000 were performed according to the Affymetrix GeneChip® Expression Analysis Technical Manual.

4.4. Quantitative PCR (qPCR)

cDNAs were synthesized from 1μg of RNA in a reaction mixture of 20 μL containing 1X PCR buffer II (Applied Biosynthesis), 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 20 units of RNase inhibitor, 50 units of murine leukemia virus reverse transcriptase (Applied Biosystems), 2.5 mM oligo(dT)18, and diethyl pyrocarbonate-treated water according to the manufacturer's instructions. Transcript levels were determined using a 7300 Real-Time PCR System (Applied Biosystems) and SYBR Green dye (Applied Biosystems). Gene specific primers (Additional file 3) were designed using the Oligo Explorer 1.2 software (Gene Link) based on the corresponding probeset sequence from the custom GrapeGen GeneChip™. No-template controls were included for each primer pair, and each PCR reaction was performed in triplicate. Data were analysed using the 7300 SDS software 1.3 (Applied Biosystems). Dissociation curves for each amplicon were then analysed to verify the specificity of each amplification reaction. Transcript level was calculated using the standard curve method and normalized against grapevine actin gene (Vvi.7514) used as reference control.

4.5. Differential gene expression

The mRNA expression profiles of the different treatments described above were compared using the Affymetrix GrapeGen custom GeneChip™ (Lijavetzky et al., *in preparation*). In total 36 samples were analysed (four berry sampling dates x 3 irrigation treatments x 3 biological replicates). Differential gene expression data analysis was done using the entire array information (23096 probesets) and this was performed using Partek Genomics Suite software. Briefly, GrapeGen GeneChip CEL files were imported by using the Robust Multichip Average method (background correction of the perfect match values, quintile normalization across all of the chips in the experiment, Log2 transformation, and median polish summarization). Log2 data were further used for statistical analysis. Differentially expressed genes were identified using ANOVA and a false discovery rate with $p<0.05$ threshold. Pearson's correlation distance based on the

gene expression profiles was used to perform a K-means cluster analysis using Genesis software (Eisen et al. 1998; Sturn et al. 2002). The present array was generated for grapevine and its design was based on the public available Unigene information at the National Center for Biotechnology Information by July 2006 (342576 grapevine ESTs). The probesets represent *V.vinifera* consensus sequences from several grape varieties (Cabernet Sauvignon, Chardonnay, Muscat Hamburg, Pinot Noir and Shiraz). It was determined that the array contains ca. 15800 unique probesets (Pontin et al. 2010). This was achieved by mapping the original 23096 probesets to the recently released 12X grapevine genomic sequence (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Still, in the present manuscript we only corrected the redundant probesets that were presented and discussed. These were merged and their average value assigned to the corresponding probeset. The functional classification of the GrapeGen GeneChip™ probesets was performed as described (Pontin et al. 2010).

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6. LIST OF ADDITIONAL FILES

Additional file 1 Genes differentially expressed during grape development and ripening under full-irrigation (FI), regulated-deficit irrigation (RDI) and non-irrigation (NI) conditions.
Additional file 2 Clustering analysis of the differentially expressed genes.
Additional file 3 qRT-PCR list of primers and expression validation.

Additional files are provided in the enclosed CD.

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Chapter III

Water deficit intensity modulates grape berry ripening- a proteomic and metabolomic analysis

Version of the manuscript entitled: *Water deficit intensity modulates grape berry ripening- a proteomic and metabolomic analysis*

Rita Francisco[1], Olfa Zarrouk[1], José António Passarinho[2], Raquen Raissa Santos[1], Joaquim Miguel Costa[1,3], Maria Fernanda Ortuño[1], Suzette Moes[4], Paul Jenöe[4], Cândido Pinto Ricardo[1,3], Maria Manuela Chaves [1,3]

[1]Instituto de Tecnologia Química e Biológica, Portugal; [2] Instituto Nacional de Recursos Biológicos, Portugal; [3]Instituto Superior de Agronomia, Portugal; [4]Biozentrum, Basel University, Switzerland

Under submission

R Francisco declares that have actively contributed to the experimental design, grape sampling, protein extraction, 2DE, bioinformatic analysis, data interpretation and manuscript writing.

ABSTRACT

Irrigation is used in viticulture to maintain yield and to improve berry quality when grapevines grow under water stress. Improving grape/wine quality under irrigation depends on a better understanding of the mechanisms that regulate berry ripening. Therefore, the effects of different irrigation strategies on berry ripening were studied by a metabolomic/proteomic approach. NMR and biochemical results show that sugars, organic acids and anthocyanins were modulated by plant water status. Mild water deficit (pre-dawn leaf water potential -Ψ_{pd}- varying between -0.41 and -0.75 MPa) induced by regulated-deficit irrigation advanced ripening-associated processes whereas moderate water deficit (Ψ_{pd} between -0.65 and -0.86 MPa) induced by non-irrigation promoted the accumulation of anthocyanins and phenols. The 2-DE/MS-MS analysis identified 74 exocarp proteins. From these, 47, 47 and 43 proteins were differentially expressed during berry ripening under control (full-irrigation), regulated-deficit irrigation and non-irrigation, respectively. Protein patterns of vacuolar invertase, flavonoid-associated and glycolytic enzymes reflect the exocarp metabolism. A set of proteins (thaumatin-like protein, ascorbate peroxidase, alcohol dehydrogenase, tubulin, ATP synthase, adenosine kinase and S-adenosylmethionine synthetase) are proposed as 'signature proteins' of the *véraison* stage. The data presented clarify the regulatory effect of plant water status on the proteins/metabolites associated with berry ripening.

Keywords: Berry exocarp, Fruit ripening, Grapevine, Proteome, Water deficit.

1. INTRODUCTION

Vineyard irrigation has become a wide spread agronomic practice in semi-dry climates. The combined effect of drought, high temperature and high evaporative demand during the growing season is known to limit photosynthesis and thereby berry productivity and quality (Escalona et al. 1999; Chaves et al. 2010; Lovisolo et al. 2010). The use of irrigation arises as a solution to overcome these deleterious effects and in more extreme cases to guarantee plant survival (Chaves et al. 2010). Paradoxically, water stress does not exclusively imply negative effects. Mild to moderate water deficit (WD), induced by deficit irrigation strategies, is intentionally applied to improve grape quality traits while maintaining yield, as it balances vegetative and reproductive growth (Chaves et al. 2010; Dry et al. 2001; Chaves et al. 2007; Santos et al. 2007). Deficit irrigation strategies, namely regulated-deficit irrigation (RDI) supplies water below full crop evapotranspiration (Et_c). Moreover, RDI withholds irrigation at key stages of fruit development/ripening aiming at an increase in the skin/pulp ratio, with likely positive effects on fruit and wine quality (MacCarthy et al. 1997; Kennedy et al. 2002). Direct effects of WD on berry metabolism have also been reported, namely on skin tannins and anthocyanins contents (Roby et al. 2004).

The physiological, biochemical and molecular mechanisms underlying grape berry ripening have been the focus of the grapevine scientific community due to the economical relevance of wine worldwide. Grape berry development follows a double sigmoid curve (Coombe 1992; Coombe and McCarthy 2000) characterized by three developmental stages: an intense cell division and expansion phase (Stage I), a transition phase where growth is reduced and finally a second growth period that involves mainly cell expansion (stage III). The transition between the first and second growth phase is called *véraison*. It marks the beginning of grape ripening and it is characterized by intense physiological changes such as fruit softening and anthocyanins accumulation. Hormonal regulation (Davies et al. 1997; Chervin et al. 2004; Symons et al. 2006; Davies and Böttcher 2009) and more recently turgor pressure (Thomas et al. 2008) have been

described as controlling many of the metabolic events that occur at the onset of ripening.

The sequence information on the entire genome of *Vitis* cv Pinot Noir and the extensive EST collection are powerful resources now available for grapevine studies. Several approaches have been exploited to understand the physiology of berry development and ripening (Davies and Robinson 2000; Deluc et al. 2007; Pilati et al. 2007). Recently, integrative '*omics'* studies proposed several proteins as putative biomarkers for grape berry ripening and post-harvest physiology (Zamboni et al. 2010; Negri et al. 2011). *Vitis* berry proteome and subproteomes (exocarp, mesocarp tissues; cell wall and plasma membrane subfractions) either at a precise stage of maturation (Sarry et al. 2004; Flamini and Rosso 2006), during berry ripening (Famiani et al. 2000; Deytieux et al. 2007; Giribaldi et al. 2007; Negri et al. 2008; Zhang et al. 2008) or post-ripening (Di Carli et al. 2010) are also important available data. Giribaldi and co-workers (2010) addressed the effects of exogenous abscisic acid (ABA) application on the grape berry proteome. It was observed that ABA acts over some of the already described ripening-related proteins. The impact of deficit irrigation on grape berry gene expression with particular relevance to quality traits, such as anthocyanins (Castellarin et al. 2007a, b) or flavour compounds (Deluc et al. 2009) were also studied. The effect of WD on the grape berry proteome is so far limited to one study carried out by Grimplet et al. (2009), where a survey of expression patterns of pericarp and seed tissue-specific proteins was performed in fully mature berries. In depth protein profiling combined with physiological and metabolomic data is likely to lead to a better understanding of the effects of WD on grape berry metabolism. The present study was undertaken to explore the potential of proteomics research into development and ripening of grape berry var. Aragonez (syn. *Tempranillo*) which is one of the most important varieties in Iberia Peninsula. Furthermore, the use of deficit irrigation as a mean to manipulate berry ripening is also explored it this study. We aim to report the effect of different water status on: 1) berry ripening with particular relevance to sugar and anthocyanins accumulation as markers of the beginning of the

ripening process and 2) the expression of proteins directly related to exocarp alterations during ripening.

2. RESULTS

2.1. Ecophysiological and biochemical characterization

2.1.1. Grapevines water status and berry growth

Leaf water potential at the pre-dawn (ψ_{pd}) was periodically monitored in FI, RDI and NI vines as an indicator of plant water status, from June to beginning of September 2006 (Figure 1). The irrigation treatments resulted in significant differences in vine water status before and after *véraison*. ψ_{pd} of the FI grapevines remained constant until maturation, when a decrease was observed due to ceasing of irrigation (from -0.22 to -0.43 MPa). ψ_{pd} of NI grapevines significantly decreased from June onwards reaching values of -0.86 MPa at the beginning of August. RDI treatment showed intermediate values compared to those observed in FI and NI, being constant throughout the season until the beginning of August (-0.41 MPa) when a significant decrease was observed (-0.75 MPa) coincident with the latter stages of maturation.

Berry growth was significantly reduced under NI treatment, at 78 and 98 DAF when compared either with FI or RDI treatments (Figure 2A).

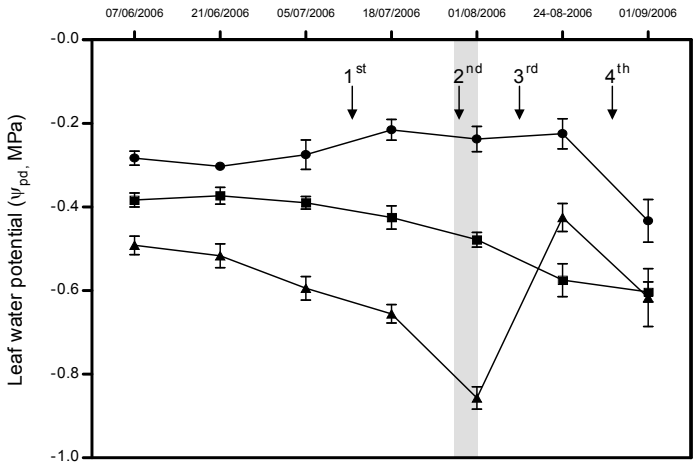

Figure 1 Pre-dawn leaf water potential (ψ_{pd}) of grapevines under different irrigation strategies.

Figure 1 Pre-dawn leaf water potential (ψ_{pd}) of grapevines under different irrigation treatments: FI (●), RDI (■) and NI (▲) during the growing season of 2006. Symbols represent means ± SE; n = 6. FI irrigation started at 29/05/06 and RDI at 16/06/06. Irrigation treatments stopped at 01/09/06. Precipitation occurred at: 12-19 June (1mm), 12-13 July (0.4mm) and 16-17 August (0.7mm). Arrows indicate the sampling dates: 07/07/06, 28/07/06, 10/10/06 and 30/10/06. Grey box represent the *véraison* period. FI, Full-irrigated; RDI, regulated-deficit irrigated; NI, non-irrigated.

2.1.2.Sugars and organic acids metabolism in the grape pericarp

Hexoses content (μmol $berry^{-1}$) and concentration (μmol g^{-1}FW) in the berry pericarp increased during fruit ripening. Moreover the rate of sugars accumulation was higher until 65DAF, under RDI conditions; the rate was intermediate under NI and lower under FI (Figure 2B,E). Malic acid decrease was rapid until 65DAF under WD being its consumption much faster in RDI than in NI (Figure 2C,F). At 98DAF no significant differences were observed either in malic or in tartaric acid between the irrigation treatments.

2.1.3.Total phenols and total anthocyanins in berry exocarp

Total phenols content (mg GAE $berry^{-1}$) increased during berry development/maturation; this trend was observed in all treatments (Figure 2D). By the other hand its concentration in the berry exocarp increased under WD, being the differences statistically significant at 44DAF and the 78DAF (Figure 2G). The onset of anthocyanins accumulation was earlier in RDI than in the other treatments, although NI berries accumulated at 98DAF higher anthocyanins concentration than either RDI or FI despite that in a per berry basis this difference was not statistically significant (Figure 2G).

2.2. Protein expression profiles during fruit maturation under different water status

2.2.1.Exocarp 2-DE reference maps

Under FI conditions the changes in the exocarp proteome that occurred during development and ripening were analysed by 2-DE. The effect of WD conditions (RDI and NI) on that proteome was also analysed. Representative gels are shown in Figure 3A.

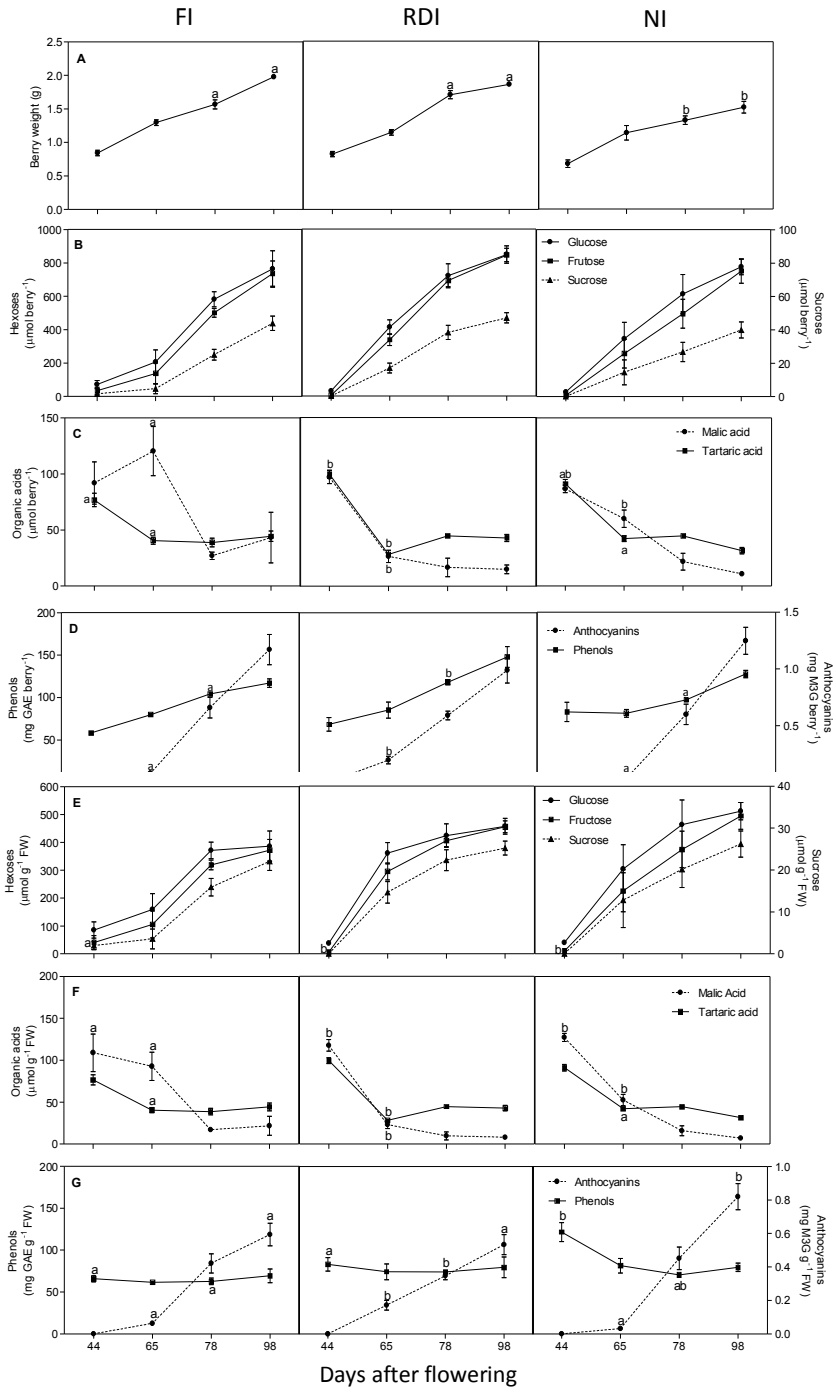

Figure 2 Grape metabolite profiling during fruit ripening. Weight (**A**) and metabolite profile (B-G) of grape berries during development and ripening and under irrigation treatments (FI, RDI and NI): 44, 65, 78 and 98 days after flowering (DAF). Content (**B-D**) and concentration (**E-F**) profiles of: **B, E**) Sugars (● glucose, ■ fructose, ▲ sucrose); **C, F**) Organic acids (● malic acid, ■ tartaric acid) and **D,G**) Anthocyanins (●) and phenols (■). Symbols represent means ± SE. Values with different letters indicate significance between irrigation treatments ($p \leq 0.05$). Left panel – FI (Full-irrigation); middle panel- RDI (regulated-deficit irrigation) and right panel-NI (non-irrigation).

The average number of detected spots did not vary significantly for the conditions studied. After performing software-assisted image analysis a total of 953 spots were taken into account in this study. These spots represent the exocarp reference gel (Figure 3B). The analysis of variance ($p \leq 0.05$) revealed 173 polypeptide spots as differentially expressed either due to berry development or plant water status. A total of 86 spots were excised from the Coomassie-blue stained gels. The identified proteins are shown in Table 1. Of the 74 identified proteins 23% can be classified as belonging to 'carbohydrate metabolism', 23% as 'stress response' related proteins, whereas 'miscellaneous', 'protein folding, stability and degradation', 'energy', represent 11% each, 'amino acids metabolism' 9%, 'flavonoids metabolism' 7% and 'cell structure and motility' 5%. From the statistical analysis, 47, 47 and 43 differentially expressed proteins during berry development were identified under FI, RDI and NI WD, respectively.

2.2.2.Hierarchical clustering analysis

To gain information on proteins with differential expression during berry development (see supporting information S1 for expression profiles of all identified proteins) a hierarchical cluster analysis was performed (Figure 4). Under FI 3 main protein clusters were observed. *Cluster I* was characterized by proteins whose expression was higher at 44DAF or 44DAF/65DAF and then decreased on the whole du ring fruit maturation, whereas *Cluster II* contains proteins whose abundance was higher at the final stages of berry ripening. *Cluster III* describes a group of proteins that were repressed only at 65DAF. Under WD conditions (RDI and NI) an additional cluster (*Cluster IV*) was observed, characterized by spots with a peak of expression at 65DAF. Of the 47 proteins whose abundance was differentially expressed during fruit development under full irrigation (FI) 14 belong to *Cluster I* and classified as belonging to: 'stress response' (29%), 'carbohydrate metabolism' (21%), '*miscellaneous'*, 'energy' and 'amino acids metabolism' (with 14% each) and 'protein folding, stability and degradation' (7%). *Cluster II*, was represented by 28 proteins belonging to: 'stress response' (29%), 'protein folding, stability and degradation' (21%), 'carbohydrate metabolism' (18%), '*miscellaneous'* (18%), 'energy' (7%), and 'amino acids metabolism' (7%). *Cluster III* was

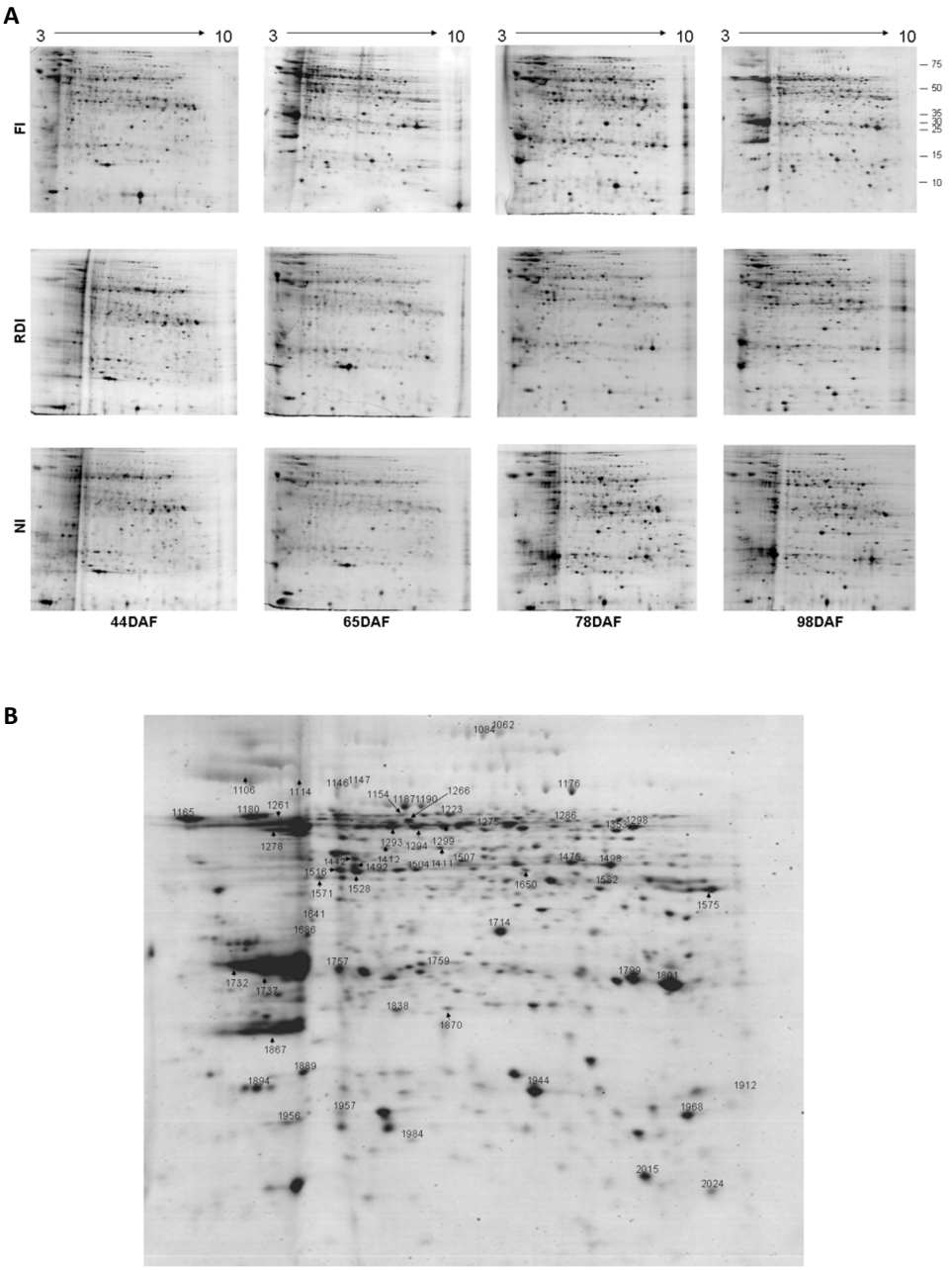

Figure 3 Representative Coomassie blue G-250 2D gels of exocarp. **A**) 2D gels of berry development and ripening (44, 65, 78 and 98 DAF) under FI, RDI and NI conditions. Proteins (250 µg) were separated by IEF using 13-cm immobilized pH gradient gels (non-linear pH 3–10), followed by a 12.5% SDS-PAGE second dimension; **B**) Exocarp reference gel with the identified MS/MS spots showing their spot ID (see Table 1 and Supporting information S1 and S2).

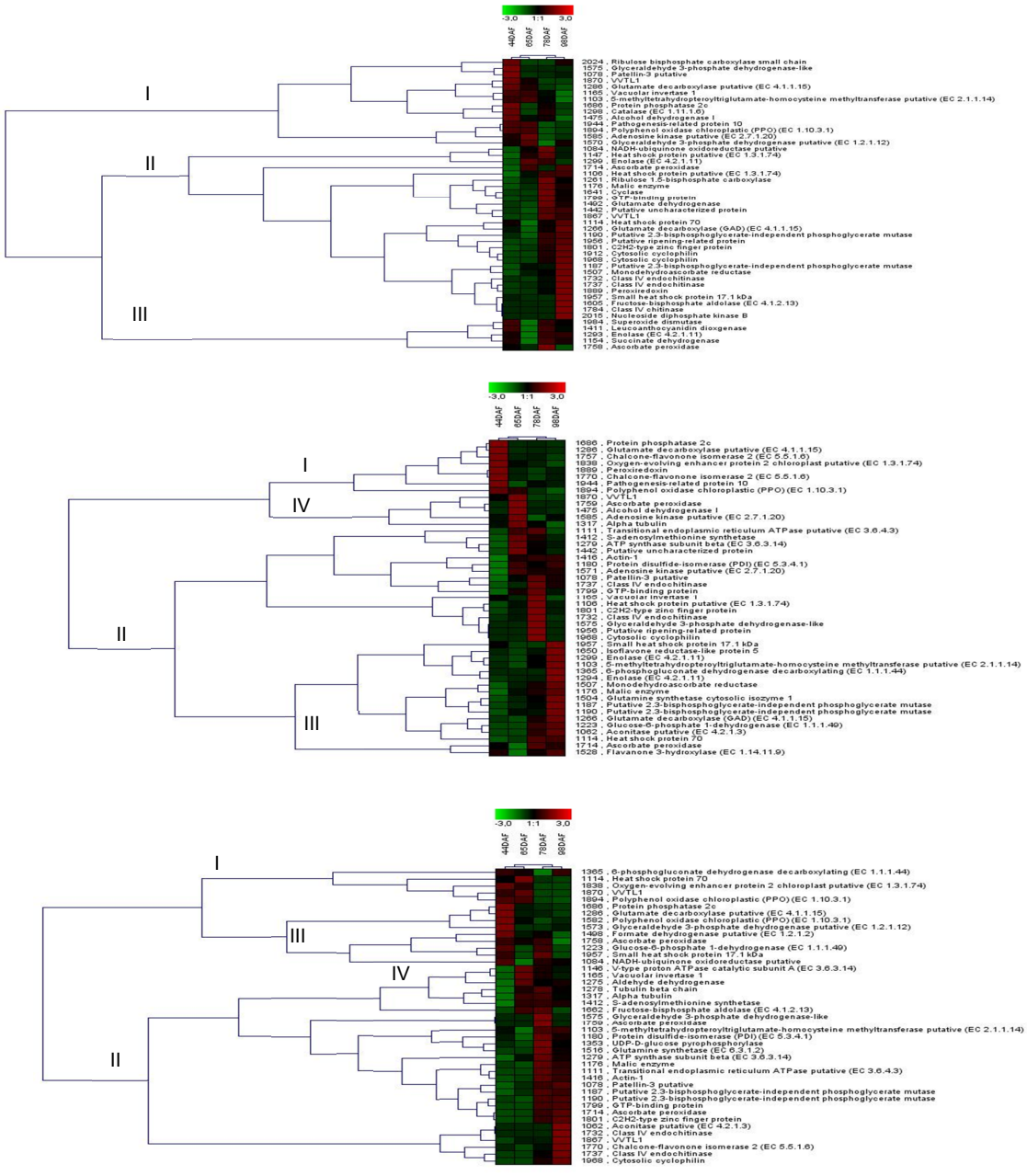

Figure 4 Hierarchical clustering analysis of the differentially expressed spots listed in Table 1. The spots were clustered according to their percentage of normalized volume from 44 to 98DAF: **A**) under FI conditions 47 proteins were grouped into 3 main clusters: I, II and III clusters; **B**) under RDI conditions 47 proteins were grouped into 4 main clusters: I, II, III and IV clusters; **C**) under NI conditions 43 proteins were grouped into 4 clusters: I, II, III and IV clusters.

composed by 5 proteins, belonging to 'carbohydrate metabolism' (40%), 'stress response' (40%) and 'flavonoids metabolism' (20%).

Of the 47 proteins considered under RDI, 8 belong to *Cluster I*: 'stress response' (38%), 'flavonoids metabolism' (25%), 'energy metabolism' (13%), 'amino acids metabolism' (13%) and 'protein folding, stability and degradation' (13%). *Cluster II* comprised 29 proteins that belonged to: 'carbohydrate metabolism' (34%), 'protein folding, stability and degradation' (17%), '*miscellaneous'* (14%), 'stress response' (14%), 'amino acids metabolism' (10%), 'cell structure and motility' (7%), and 'flavonoids metabolism' (3%). Two proteins belonged to *Cluster III* classified as 'flavonoids metabolism' and 'stress response'. *Cluster IV* was composed by 8 proteins that belonged to: 'energy metabolism' (25%), '*miscellaneous'* (25%), 'stress response' (25%) 'amino acids metabolism' (13%) and 'cell structure and motility' (13%).

Of the 43 proteins considered under NI, *Cluster I* was composed by 9 proteins related to: 'stress response' (32%), 'carbohydrate metabolism' (22%), 'protein folding, stability and degradation (22%), 'amino acids metabolism' and 'energy metabolism' (11% each). *Cluster II* comprised 26 proteins classified as belonging to: 'carbohydrate metabolism' (27%), 'stress response' (19%), 'cell structure and motility' (15%), 'amino acids metabolism' (12%), '*miscellaneous'* (12%), 'protein folding, stability and degradation (8%), 'energy' (4%) and 'flavonoids metabolism (4%). *Cluster III* was composed by 5 proteins classified as: 'energy' (40%), 'carbohydrate metabolism', 'protein folding, stability and degradation' and 'stress response' (20% each). Cluster IV grouped 3 proteins that belonged to: 'carbohydrate metabolism', 'energy metabolism' and 'stress response'.

3. DISCUSSION

3.1. Berry development/ripening of FI grapevines

Under FI conditions the accumulation of sugars and anthocyanins and the metabolization of malic acid was most significant from 65DAF onwards (see Figure 2), suggesting that *véraison* occurred later than the second sampling date (65DAF). The

changes observed in protein expression (see Figure 4 and supporting information S1) were interpreted in view of these metabolic trends. At 65DAF, vacuolar invertase (GIN1) attains its maximal expression being progressively down-regulated thereafter, which agrees with the expression of *GIN1* in grape berries preceding hexoses accumulation. Nevertheless, enzyme activity remained somewhat constant throughout berry maturation (Davies and Robinson 1996). A general trend of glycolysis activation was observed throughout berry ripening, as several glycolytic enzymes such as phosphoglycerate mutase (PGAM#1187, #1190), enolase (ENO#1299) and aldolase (ALDO#1605) were progressively increasing. This confirms previous data (Negri et al. 2008; Di Carli et al. 2010). Malic enzyme (ME) also increased at later stages of maturation. Grimplet *et al.* (2007) reported that in the exocarp, transcripts related to malate metabolism remain highly expressed. From the enzymes involved in the anthocyanins pathway only leucoanthocyanidin dioxygenase (LDOX) was shown to be differentially expressed throughout development under FI conditions. LDOX was down-regulated at 65DAF followed by an increase at latter stages of ripening. Several studies reported this bimodal pattern of gene expression for some flavonoid genes, *LDOX* included (Castellarin et al. 2007a,b; Boss et al. 1996; Bogs et al. 2005). Alcohol dehydrogenase (ADH1) was also identified and its expression profile decreased during fruit maturation. ADH belongs to a multigenic family and in *V.vinifera Adh1, Adh2 and Adh3* genes are the best characterized during berry development and ripening. Vv*Adh1, VvAdh3* are expressed up to *véraison* and down-regulated thereafter, whereas Vv*Adh2* is up-regulated during ripening (Tesnière et al. 2000).

3.1.1. The expression profile of heat-shock proteins, cyclophylins and phosphatase 2C during fruit maturation

In our study, the expression of HSP70 (#1106, #1114) and HSP17 increased at later stages of berry maturation. Different evolution patterns of these proteins during berry metabolism have been described (Deytieux et al. 2007; Giribaldi et al. 2007; Di Carli et al. 2010; Negri et al. 2011) what suggests that different classes of chaperones can

Table 1 Functional classification of grape exocarp proteins whose expression significantly varied during berry development and ripening (See also supporting information S2).

Spot ID[a]	Assignment	Vitis 12x[b]	Uniprot[c]	Spot name	pI (Exp/Th)[d]	MW (Exp/Th)[d]	Score XC	Cov (%)[e]
Amino acids metabolism								
1412	S-adenosylmethionine synthetase		P93254	SAM	5.26 / 5.43	50.99 / 42.89	20.15	5
1103	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase putative (EC 2.1.1.14)	GSVIVT00029971001	B9RQ33	MET	6.70 / 6.09	75.06 / 84.59	34.17	7
1266	Glutamate decarboxylase (EC 4.1.1.15)	GSVIVT00000391001	P54767	GAD	5.40 / 5.97	59.30 / 56.79	48.21	7
1286	Glutamate decarboxylase putative	GSVIVT00035247001	B9SR96	GAD	6.77 / 5.91	58.46 / 56.54	80.18	15
1492	Glutamate dehydrogenase	GSVIVT00033402001	Q1HDV6	GDHB	4.94 / 6.28	47.36 / 44.69	30.16	6
1504	Glutamine synthetase cytosolic isozyme 1	GSVIVT00008483001	P51118	GS1-1	5.59 / 5.79	45.69 / 39.20	50.16	9
1516	Glutamine synthetase (EC 6.3.1.2)	GSVIVT00018781001	Q93XJ6	GS1-1	4.80 / 5.40	44.55 / 38.96	30.19	7
Carbohydrate metabolism								
1570	Glyceraldehyde 3-phosphate dehydrogenase putative	GSVIVT00031272001	Q2XQF4	GAPDH	7.99 / 7.06	41.24 / 32.16	18.23	10
1573	Glyceraldehyde 3-phosphate dehydrogenase putative	GSVIVT00007521001	A2ICC7	GAPDH	7.82 / 7.96	41.24 / 26.31	70.16	14
1575	Glyceraldehyde 3-phosphate dehydrogenase-like		Q2XPW9	GAPDH	9.04 / 6.34	40.28 / 36.68	16.15	6
1605	Fructose-bisphosphate aldolase (EC 4.1.2.13)	GSVIVT00033791001	Q2WFK8	ALDO	7.60 / 8.87	35.90 / 36.49	70.21	19
1662	Fructose-bisphosphate aldolase	GSVIVT00011810001	B9SJY9	ALDO	9.04 / 8.71	38.93 / 39.99	20.23	7
1187	Putative 2.3-bisphosphoglycerate-independent phosphoglycerate mutase	GSVIVT00029842001	C5DB50	PGAM	5.49 / 5.38	65.00 / 53.40	100.24	17
1190	Putative 2.3-bisphosphoglycerate-independent phosphoglycerate mutase	GSVIVT00029842001	C5DB50	PGAM	5.65 / 5.36	65.00 / 62.67	110.26	22
1293	Enolase (EC 4.2.1.11)	GSVIVT00033770001	Q9LEI9	ENO2	5.32 / 5.67	57.72 / 47.87	50.26	14
1294	Enolase	GSVIVT00033770001	Q9LEI9	ENO2	5.57 / 5.62	57.73 / 48.35	138.26	34
1299	Enolase	GSVIVT00033770001	Q9LEI9	ENO	5.80 / 7.53	57.62 / 48.03	138.26	31
1223	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	GSVIVT00001847001	Q2XTC4	G6PD	5.81 / 5.97	62.59 / 58.49	60.14	11
1365	6-phosphogluconate dehydrogenase decarboxylating	GSVIVT00010170001	B9SXT4	6PDG	4.73 / 8.13	50.53 / 48.29	50.19	9
1062	Aconitase putative (EC 4.2.1.3)	GSVIVT00037657001	B9SDW5	ACO	5.99 / 7.11	83.61 /108.79	40.13	4
1154	Succinate dehydrogenase	GSVIVT00018976001	B9SWW3	SDH1-1	5.57 / 7.11	67.38/108.79	60.20	10
1353	UDP-D-glucose pyrophosphorylase	GSVIVT00026563001	D2D2Z1	UGP	7.77 / 5.62	54.30 / 51.32	160.25	16
1165	Vacuolar invertase 1	GSVIVT00018625001	Q9S944	GIN1	3.60 / 4.60	67.38 / 71.54	70.22	7

Cont. Table 1

1176	Malic enzyme	GSVIVT00015311001	P51615	ME	6.86 / 6.46	65.71 / 70.67	76.17	7
Cell structure and motility								
1317	Alpha tubulin	GSVIVT00037046001	B7TIW7	TubA	4.03 / 4.76	80.80 / 69.92	38.20	11
1278	Tubulin beta chain	GSVIVT00019758001	B9GWI1	TubB	4.18 / 4.77	57.93 / 43.62	98.22	14
1416	Actin-1	GSVIVT00016550001	Q10DV7	ACT1	7.52 / 5.30	53.91 / 41.81	120.23	30
1111	Transitional endoplasmic reticulum ATPase putative	GSVIVT00025723001	B9S0I1		4.52 / 5.27	58.77 / 45.29	110.28	8
Energy metabolism								
1838	Oxygen-evolving enhancer protein 2 chloroplast putative	GSVIVT00020818001	Q6XNL9	PSBP1	4.98 / 8.05	24.00 / 10.30	70.24	35
1261	Ribulose 1.5-bisphosphate carboxylase	GSVIVT00022403001	P56648	RBCL	3.99 / 6.17	59.62 / 47.46	60.20	10
2024	Ribulose bisphosphate carboxylase small chain	GSVIVT00008288001	Q2I314	RBCS1-1	8.93 / 9.23	6.75 / 20.03	50.20	33
1084	NADH-ubiquinone oxidoreductase putative	GSVIVT00030914001	B9T118	CI-75kD	5.88 / 5.77	80.80 / 68.43	120.24	27
1498	Formate dehydrogenase putative (EC 1.2.1.2)	GSVIVT00032479001	B9RUT7	FDH	7.44 / 6.28	45.86 / 42.61	130.22	7
1279	ATP synthase subunit beta (EC 3.6.3.14)	GSVIVT00011797001	C6GFP3	ATPB	4.33 / 4.93	56.90 / 49.57	120.27	33
1146	V-type proton ATPase catalytic subunit A (EC 3.6.3.14)	GSVIVT00029546001	P09469	V-ATPase A	4.77 / 5.29	67.50 / 68.68	194.24	23
1475	Alcohol dehydrogenase I	GSVIVT00010024001	Q43690	ADH1	6.78 / 6.08	47.19 / 41.13	90.17	23
Flavonoids metabolism								
1757	Chalcone-flavonone isomerase 2 (EC 5.5.1.6)	GSVIVT00032619001	P51117	CHI2	4.72 / 5.26	28.68 / 25.14	90.22	36
1770	Chalcone-flavonone isomerase 2	GSVIVT00032619001	P51117	CHI2	5.00 / 5.53	27.71 / 25.73	60.20	25
1528	Flavanone 3-hydroxylase (EC 1.14.11.9)	GSVIVT00018781001	A2ICC8	F3H	4.97 / 5.44	44.16 / 40.87	80.21	22
1650	Isoflavone reductase-like protein 5	GSVIVT00023800001	Q3KN68	IFRL5	6.32 / 5.50	36.95 / 33.84	20.17	4
1411	Leucoanthocyanidin dioxgenase	GSVIVT00019892001	Q8LP73	LDOX	5.80 / 6.30	51.26 / 41.04	70.23	21
Miscellaneous								
1571	Adenosine kinase putative (EC 2.7.1.20)	GSVIVT00016250001	B9T0A9	ADK	4.36 / 5.01	41.09 / 37.57	110.27	15
1585	Adenosine kinase putative	GSVIVT00016250001	B9T0A9	ADK	4.74/5.01	40.58/37.57	20.16	8
2015	Nucleoside diphosphate kinase B	GSVIVT00018297001	Q6Y0E7	NDKB	7.67 / 9.08	8.06 / 20.86	40.26	25
1799	GTP-binding protein		Q8S2Z7	RAN	7.68 / 6.39	26.11 / 25.09	18.13	10
1801	C2H2-type zinc finger protein		Q93XJ4	LIF	8.30 / 7.76	25.56 / 23.49	10.17	8
1078	Patellin-3 putative	GSVIVT00026077001	B9S732	PATL	4.67 / 5.13	72.79 / 89.61	30.15	10
1641	Cyclase	GSVIVT00003172001	Q2I313		4.41/ 8.90	37.02 / 36.40	30.16	7
1442	Putative uncharacterized protein	GSVIVT00035803001	O24329		4.93 / 7.57	49.19 / 40.01	30.10	6
Protein folding, stability and degradation								

Cont. Table 1

1106	Heat shock protein putative (EC 1.3.1.74)	GSVIVT00008331001	B9SKC7	HSP70	3.86 / 5.22	71.63 / 75.45	190.26	15
1114	Heat shock protein 70	GSVIVT00011728001	Q9M4E6	HSP70	4.39 / 5.26	70.48 / 70.52	80.21	12
1147	Heat shock protein putative (EC 1.3.1.74)	GSVIVT00038517001	B9RX55	Hsp70	4.97 / 5.87	67.87 / 72.35	70.24	9
1957	Small heat shock protein 17.1 kDa	GSVIVT00016428001	D1MIX5	Hsp17	5.15 / 5.82	11.69 / 18.19	36.20	18
1912	Cytosolic cyclophilin	GSVIVT00009919001	A5AKD8	PPIase	8.99 / 8.71	12.68 / 18.29	30.15	41
1968	Cytosolic cyclophilin		O49886	PPIase	8.41 / 8.72	11.04 / 18.29	20.23	7
1180	Protein disulfide-isomerase (PDI) (EC 5.3.4.1)	GSVIVT00008848001	Q9XF61	PDI	4.14 / 4.84	65.00 / 57.09	250.23	45
1686	Protein phosphatase 2c	GSVIVT00036034001	B9RNR4	PP2	4.46 / 6.48	34.51 / 35.13	60.23	23
Stress response								
1714	Ascorbate peroxidase		Q1AFF4	APX	5.90 / 5.58	31.55 / 27.62	28.16	17
1758	Ascorbate peroxidase	GSVIVT00025104001	A2T400	APX	5.60 / 5.58	28.88 / 27.62	30.18	19
1759	Ascorbate peroxidase	GSVIVT00025104001	A2T400	APX	5.82 / 5.11	28.94 / 20.36	48.16	12
1298	Catalase (EC 1.11.1.6)	GSVIVT00002361001	Q8S568	CAT	8.23 / 6.90	58.04 / 56.97	30.21	7
1507	Monodehydroascorbate reductase	GSVIVT00003351001	A5JPK7	MDAR	5.85 / 5.93	45.69 / 47.28	60.20	17
1984	Superoxide dismutase	GSVIVT00021959001	Q70CE4	SOD	5.49 / 5.71	10.01 / 15.23	30.14	24
1889	Peroxiredoxin		Q8S3L0	PRx	4.42 / 5.56	15.97 / 17.43	10.16	6
1582	Polyphenol oxidase chloroplastic (PPO) (EC 1.10.3.1)	GSVIVT00006794001	Q9SPF6	PPO	7.37 / 6.27	40.29 / 67.35	40.20	7
1894	Polyphenol oxidase chloroplastic	GSVIVT00006794001	Q9SPF6	PPO	4.14 / 6.27	15.39 / 67.35	40.20	7
1732	Class IV endochitinase		O24530	VvChi4A	3.81 / 5.38	30.16 / 27.24	20.21	17
1737	Class IV endochitinase		O24530	VvChi4A	3.98 / 5.38	30.16 / 27.24	10.21	9
1784	Class IV chitinase	GSVIVT00038125001	Q7XAU6	Chi4D	7.91 / 5.38	27.41 / 27.53	30.20	7
1867	VVTL1	GSVIVT00019848001	O04708	VVTL1	3.80 / 5.31	20.70 / 24.32	40.21	18
1870	VVTL1	GSVIVT00019848001	O04708	VVTL1	5.32 / 5.31	21.22 / 24.32	10.24	11
1944	Pathogenesis-related protein 10		Q9FS43	PR10.2	6.10 / 5.96	12.71 / 17.13	40.18	28
1275	Aldehyde dehydrogenase	GSVIVT00020224001	Q1AFF6	ALDH2a	5.85 / 8.04	59.09 / 58.52	90.29	17
1956	Putative ripening-related protein	GSVIVT00011728001	Q9M4G9	Grip61	4.31 / 5.12	11.85 / 17.08	80.19	25

[a)] Spot ID numbers identified in 2-DE gel presented in Figure 3B; [b)] Protein match from the Vitis 12x genome annotation (EnsemblPlants database)

[c)] Uniprot ID of the most closely related protein from GSVIV; [d)] Exp/Th, experimental and theoretical determination of pI and molecular mass (MW)

[e)] % coverage

intervene in different stages of berry maturation and/or different berry tissues. Two cyclophylins (PPIase, #1912, #1968) significantly increased during berry development reaching its maximum 98DAF. These proteins apart from fulfilling the basic role of protein folding, may also be involved in other important roles such as mRNA processing, protein degradation, and signal transduction during development and stress responses (Romano et al. 2004). Phosphatase 2c (PP2) was also identified with its maximal spot intensity observed at 44DAF. This class of phosphatases has been implicated in a variety of processes e.g. signalling transduction and hormonal control, particularly as ABA negative regulator (Schweighofer et al. 2000). More recently, Gambetta et al. (2010) proposed several novel candidates, PP2 included, in the control of grape ripening, as part of ABA/sugar signalling pathways. However, the expression pattern that we observed is not consistent with the ABA regulator role, since a sharp increase of ABA concentration is observed around véraison, which is followed by a decrease during grape ripening (Davies and Böttcher 2009).

3.1.2.Stress-related proteins and grape ripening

Stress-related proteins also have a great contribution to grape berry development and maturation (Davies and Robinson 2000; Deytieux et al. 2007; Giribaldi et al. 2007; Negri et al. 2008). Considering their expression patterns two major groups could be described: those belonging to Cluster I and those that belong to Cluster II. In the first group a thaumatin-like protein (TL1#1870), a pathogenesis-related protein 10 (PR10.2), a polyphenol oxidase (PPO #1894), and a catalase (CAT) were detected, whereas ascorbate peroxidase (APX #1714), glutathione reductase (GR), monodehydroascorbate reductase (MDAR), chitinase class IV (Chi4A #1732, #1737, Chi4D#1784), TL1 (#1867) and a putative-ripening related protein (Grip61) could be included in the second cluster. The activation of enzymes that mediate reactive-oxygen species (ROS) scavenging, like APX, GR, and MDAR suggests that an intensification of oxidative stress may occur at the later stages of berry ripening as already observed (Pilati et al. 2007). Pathogenesis related (PR) proteins are not only the most abundant proteins in grape berries (Salzman et al. 1998; Pocock et al. 2000) but are also highly expressed throughout various stages

of berry growth (Deluc et al. 2007), as it was observed in the present study. The synchronized accumulation of PR proteins and hexoses seems to constitute a developmentally regulated defence mechanism against fungi in the mature fruit (Robinson et al. 1997; Tattersall et al. 1997; Salzman et al. 1998).

3.1.3.New players of the ripening process

Patellin-3 (PATL) was also identified in this study and it significantly decreased 65DAF, after which its expression remained constant throughout berry maturation. In agreement, Zhang et al. (2008) observed that in Cabernet Sauvignon this protein was expressed at 50, 75 and 95DAF (before, at and after *véraison* respectively). PATL is a carrier protein that is involved in membrane trafficking events namely the transference of hydrophobic molecules such as phosphoinositides (Peterman et al. 2004). It was suggested that it is involved in active cellular processes during the intense metabolic alterations that occur during grape berry maturation (Zhang et al. 2008). Other relevant proteins were the GTPase RAN-A1 and the lateral shoot-inducing factor l (LIF). RAN-A1 expression significantly increased from 65DAF on. Small GTPases have been described as molecular switches that regulate several cellular processes such as vesicle trafficking, cytoskeletal dynamics, cell polarity and gene expression (Yang 2002). Zamboni *et al.* (2010) proposed a GTP-binding nuclear protein Ran-3 transcript as a biomarker of the post-harvest berry stage. The role of grape GTPase genes in vesicular transport during ripening was also studied (Abbal et al. 2007, 2008). It seems that a coordinated expression of a set of genes encoding small GTPases families takes place during key moments of fruit development and maturation (Falchi et al. 2010). LIF expression was also associated with the ripening process. Described in petunia as a zinc-finger protein, its overexpression was responsible for morphological alterations associated with altered cytokinins metabolism (Nakagawa et al. 2004). Thus, although no members of this gene family have been described to have a function in fruit ripening the data presented may suggest such participation.

3.2. Effect of water deficit on berry development and ripening

The results showed that WD influenced the development and maturation pattern of grape berry. Several parameters that constitute the suite of changes of berry ripening were in fact altered. In RDI and NI conditions, sugars accumulation and organic acids metabolization started earlier when compared to FI, these features being more evident under RDI conditions. RDI advanced anthocyanins accumulation, although their concentration attained the maximum value under NI conditions (at 98DAF). Esteban et al. (2001) reported also in Tempranillo (syn. Aragonez) that total anthocyanins concentration was significantly higher in NI than in irrigated grapes. The number of collected samples in this study imposes some limitations on data interpretation, particularly in the case of berry growth. Nevertheless, some interesting observations could be made. Under NI, berry weight was reduced at 78 and 98DAF. Moreover, in RDI the rate of berry growth was higher between stages 65 and 78DAF, suggesting that the transition to the second growth period also starts earlier when compared with both FI and NI conditions. Altogether, these results make clear that under RDI the onset of ripening occurred earlier in time (around 65DAF). Grapevines vigour, and therefore source/sink relations may have been altered into a different extent by RDI or NI, what could explain the observed differences on berry metabolism. Contradictory results on WD effects on grape ripening have been reported (Castellarin et al. 2007a; Deluc et al. 2009; Gambetta et al. 2010); this may be explained by distinct environmental conditions, viticultural practices, or varieties (Gaudillère et al. 2002; Deluc et al. 2009). Although several studies in WD effects in Tempranillo have been published in the last years (Esteban et al. 1999, 2001; Intrigliolo and Castel 2010) to the best of our knowledge our study is the first that analyses the impact of WD on grape berry ripening connecting the physiological status of the plants with the biochemical/molecular alterations at the berry level.

3.2.1.‘*Signature proteins’ of véraison stage*

The detected changes on the protein patterns reflect ripening anticipation under RDI. Indeed, the fact that a group of proteins (SAM, ADH1, TubA, TL1#1870, APX#1759, ATPB,

ADK) were specifically up-regulated at 65DAF supports such anticipation. These proteins have several roles in grape metabolism. SAM is indirectly involved in polyamines and ethylene biosynthesis (Yang and Hoffman 1984); ADH1 apart from its involvement in plants response to stressful conditions, and its responsiveness to several signals such as hormones (Tesnière et al. 2004; Giribaldi et al. 2010) is expressed in a developmentally-regulated manner, particularly during fruit ripening. The fact that ADH1 showed this expression pattern under RDI conditions may contradict the available data. However, initial reports stated that VvADH1 was up-regulated at the inception of ripening (Sarni-Manchado et al. 1997). Sarry et al. (2004) also stressed how complex it can be to precisely determine ADH isoforms in 2DE gels. TubA protein profile is consistent with the intense cell growth that occurs at the inception of *véraison*; TL1 has been proposed not only as a defence protein but as well as ripening-related; APX is related with oxidative stress; ATPB, is associated with ATP production; ADK fulfils basic metabolic roles, such as the regulation of adenine and adenosine and contributes to the interconversion of cytokinin ribosides and nucleotides (Tesnière et al. 2004). Finally, an unknown protein (#1442) was also up-regulated at 65DAF. This protein is characterized by the presence of DUF642 and galactose-binding domains.

3.2.2. The onset of ripening and the expression profiles of vacuolar invertase and enzymes related to anthocyanins biosynthesis

GIN1 was down-regulated at 44DAF as compared to FI conditions, what may be directly related with the onset of berry ripening. Invertases are key metabolic enzymes providing growing tissues with hexoses, generating a sucrose concentration gradient between source/sink tissues supporting sucrose transport into storage organs such as fruits or regulating cell turgor (Tang et al. 1999; Roitsch et al. 2004). Cell wall invertase increased in grape berry at the early stage of maturation (Zhang et al. 2006), whereas GIN1 was down-regulated during the same period (Davies and Robinson 1996; Deluc et al. 2007; Negri et al. 2008, 2011). It has been proposed that this is the result of sucrose hydrolysis shift from vacuolar to apoplastic (Zhang et al 2006). Also, vacuolar invertase seems to be under sugar and ABA crosstalk regulation (Trouverie et al.2004). ABA is not only the

major operating signal during drought stress, but in coordination with sugars may have a role in grape fruit development and ripening (Carrari et al. 2004; Gambetta et al. 2010). From 65DAF onwards NI conditions was responsible for a higher F3H and CHI expression (both #1757, #1770) as compared to RDI or FI conditions, suggesting that these variations could be related with the observed pattern of anthocyanins accumulation. In fact, Castellarin et al. (2007b) showed that total anthocyanins content correlated with the *CHS or F3H* expression. The early sugar accumulation observed under RDI may be correlated with the onset of anthocyanins biosynthesis. Castellarin *et al.* (2007a) observed the coordination between the beginning of sugar accumulation and the increase in anthocyanins-related transcripts. In addition, anthocyanins biosynthesis in grape berries was proven to be developmentally triggered in a sugar-dependent manner (Gollop et al. 2001). In our study, we detected at the latter stages of maturation a relationship between the amount of anthocyanins accumulated and the corresponding biosynthetic enzymes. However, at 65DAF such a relationship is not evident. All in all, these observations sustain the hypothesis that the signal(s) that may be required for the onset of anthocyanins accumulation are already present in RDI berries whereas in NI they appear later in time, supporting the anticipation of *véraison* under RDI conditions.

3.2.3. Major effects of water deficit on pathogenesis-related proteins, chaperones and phosphatase 2c

PR10.2 was down regulated by WD at the earlier stages of berry ripening. Described as being multifunctional, PR-10 proteins can bind ligands such as flavonoids, fatty acids or plant hormones (Carrari et al. 2004; Fernandes et al. 2009), such as cytokinins (Krishnaswamy et al. 2008). Recently, Muñoz et al. (2010) proposed that PR-10 may be related to anthocyanins biosynthesis in strawberry. In contrast to our results PR-10 was shown to be over-expressed at maturation stage (Deytieux et al. 2007; Negri et al. 2008). Although we have no explanation to this discrepancy, it can be hypothesised that different PR-10 isoforms may be subjected to different regulatory mechanisms during fruit ripening, as already observed in strawberry (Muñoz et al. 2010). TL1 (#1870) was down-regulated at later stages of berry maturation under FI and NI conditions, whereas

under RDI a peak of expression was observed at 65DAF as previously discussed. Contrarily to expectations that NI would increase TL1 expression, it was shown at comparable grape hexose content that RDI berries express more TL1 than NI berries. Three Chi4 isoforms were up-regulated during berry maturation. These results are in accordance with the observation that constitutive expression of class IV chitinase coincides with grape ripening, as chitinase activity increases (Robinson et al. 1997; Deytieux et al. 2007). The suggestion that during ripening due to sugars and other metabolites accumulation, berries become highly susceptible to pathogens favours that the induction of PR proteins may occur developmentally rather than pathogen/stress related. Both TL1 and Chi4 expression patterns do not directly reflect the intensity of the stress that vines are exposed to, but seem to be more correlated with the pattern of hexose accumulation. Interestingly, Zamboni et al. (2010) established a positive correlation between glycosylated anthocyanins and Chi4 and TL1 during ripening and post-harvest events. HSP70 (#1106) and HSP17 were overexpressed under WD conditions, HSP70 after 65DAF and HSP17 under NI already at 44DAF.

First described as induced by heat stress, HSPs are essential for protein folding, unfolding, and transport. In tomato fruit they are involved in the maturation of protein complexes and degradation of damaged or misfolded peptides and in regulating the activity of many signal transduction peptides (Neta-Sharir et al. 2005; Faurobert et al. 2007). PP2 profile was repressed under RDI or NI conditions during grape berry, but at 44DAF PP2 attained its maximum intensity under RDI. Although it is tempting to correlate this pattern to ripening anticipation that was observed under RDI conditions, as already discussed in section 3.1.1 this protein may be involved in other functions than as an ABA-regulator.

3.2.4. Water deficit intensifies oxidative stress during berry ripening

PPO expression (#1894) was highest in NI and decreased in RDI and furthermore in FI, paralleling the higher concentration of phenolic compounds in WD plants as compared to controls. High levels of PPO expression were found in developing berries (Dry and Robinson 1994), and it has been described as abundantly expressed in exocarp (Negri et

al. 2008). This pattern was also observed in other fruits (Chevalier et al. 1999; Gooding et al. 2001), suggesting a developmental role for PPO during fruit maturation (Mayer 2006). The activation of ROS scavenging enzymes such as APX (#1714; #1758) was also enhanced by NI conditions. A peak of expression of APX (#1759) was detected either at 65DAF or at 78DAF under RDI or NI conditions, respectively. This suggests that WD intensifies oxidative stress during berry ripening and ABA may be involved in such intensification (Giribaldi et al. 2010).

3.2.5. Water deficit, ripening-related proteins and hormone signalling

A group of three proteins, RAN-A1, LIF and PPIase showed down-regulation of its expression as compared to FI conditions at 78 or 98DAF. Although the function of these proteins still remains to be elucidated, their possible involvement in grape ripening could be speculated. In fact, a Ran-binding protein whose expression was reduced following ABA treatment was identified in berry skin at *véraison* (Giribaldi et al. 2010), suggesting the hormonal control of these proteins (Peterman et al. 2004). Moreover, LIF was associated with cytokinins metabolism in petunia (Falchi et al. 2010). Finally, a role of PPIase in auxin signalling and protein degradation has been proposed (Kouri et al. 2009; Oh et al. 2006), although the role of endogenous auxin in grape ripening is still unclear.

In conclusion, the present study showed that WD modulated grape berry sugars, organic acids and anthocyanins metabolism. It seems that the early hexose accumulation was a crucial feature for the alterations observed, and this was markedly relevant under RDI conditions. To function as a signal, hexose concentration must be above a certain threshold level (Herbers et al. 1996). RDI conditions advanced the onset of ripening. At the protein level some of the observed alterations were already detected at the berry green stage, underlying the importance of *pre-véraison* WD as a way of promoting an enhancement of berry quality traits, in addition to the reported alterations in berry size (MacCarthy 1997; Kennedy et al. 2002). The dynamics of protein expression patterns reflects ripening anticipation in RDI vines. A thaumatin-like protein, an ascorbate

peroxidase, an alcohol dehydrogenase, a tubulin, an ATP synthase, an adenosine kinase, an uncharacterized protein and an S-adenosylmethionine synthetase can be proposed as 'signature proteins' of the *véraison* stage. The expression profiles of proteins such as RAN-A1, LIF, and PPIase could also suggest they have a role as ripening-related proteins. It should be emphasized that the majority of the identified proteins have been described in other proteome *Vitis* cultivars. However, we provide new entries in grape berry proteome catalogue. Still a question that needs to be addressed is whether the observed trends are cultivar dependent. In a Pinot Noir study (Negri et al. 2011) several proteins (mostly stress-related) were proposed as biomarkers for ripening. Several of these proteins are described in our study, but in some cases the described trend is different. Our study opens new avenues for future research on the role of several proteins in grape ripening, as a number of observations suggest their interaction with some of the already known regulatory signals (hexoses, ABA, cytokinins).

4. MATERIALS AND METHODS

4.1. Grape irrigation treatments

Irrigation treatments were imposed during 2006 season in the commercial vineyard Monte dos Seis Reis (Estremoz, Portugal). Five year-old *Vitis vinifera* cv. Aragonez plants (grafted on 1103 Paulsen rootstock) were trained on a bilateral *Royat Cordon* system. Plants were drip irrigated at both sides of the row with 2 Lh^{-1} drippers supplying either 100% Etc (Full irrigation, FI) or 40% Etc (Regulated-deficit irrigation, RDI). Irrigation in RDI started from beginning of June until 90% of *véraison* was attained, whereas FI vines started to be irrigated from end May until beginning of September. Non-irrigated (NI) but rain-fed grapevines were also studied. FI can be referred as control condition, whereas RDI and NI as mild and moderate WD, respectively. Leaf ψ_{pd} were measured with a Scholander-type pressure chamber (Model 1000; PMS Instrument Company, Corvallis OR, USA) from the beginning of berry development until harvest.

4.2. Berry sampling

Berries were collected from the pea size until full maturation was attained, corresponding the sampling dates to 44, 65, 78, 98 days after flowering (DAF). *Véraison* was considered when 50% of the berries were coloured. At the reported days four biological replicates per treatment (composed by 15 clusters each) were harvested, at midday in both sides of the vines, immediately immersed in liquid nitrogen and stored at -80°C until use.

4.3. Metabolite analysis

4.3.1. Sugars and organic acids

Frozen berries (10 per replicate; 3 replicates were analysed) were ground in liquid nitrogen to a fine powder and dropped into boiling water (for 5 min) for enzymes inactivation. The slush was filtered through ten layers of gauze and the resultant liquid adjusted to pH 6.0 with NaOH, and then lyophilized. The lyophilized residue was solubilized in 2-3mL of a solution containing 5.8M D_2O, 2.5mM Na_2EDTA and 2.5mM NaN_3. The qualitative and quantitative characterization of the water soluble metabolites in the extracts was carried out by ^{1}H and ^{13}C-nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR), using dioxan as an internal concentration standard (23.43mM). All experiments were performed with a Bruker Avance II^{+} 400 spectrometer (Germany) operating at ^{1}H frequency of 400.13 MHz and ^{13}C frequency of 100.61 MHz, using a 5 mm diameter broadband probe head. ^{1}H-NMR spectra were acquired using a zg pr pulse sequence with the following parameters: number of scans 48, a recycle time of 5.48 s, acquisition of 64 k and a pulse angle of 30°. Data were processed with 0.3 Hz exponential line broadening. ^{13}C-NMR spectra were acquired using a zg ig pulse program with inverse gated decoupling of protons, with a total of 750 scans collected into 64 k points over a spectral width of 24 kHz, a recycle time of 3.36 s, and a pulse angle of 30°. Spectra with total relaxation were acquired with a recycle time of 31.36 s and a pulse angle of 90°. The temperature of the probe head was kept at 298 K. Data were processed with 4 Hz exponential line broadening. Chemical shifts are expressed in ppm relative to DSS (sodium 3-trimethylsilyl-1-propanesulfonate) at a final concentration of 0.0176%.

Resonances due to fructose, glucose, sucrose, malic and tartaric acids were identified from the chemical shifts of pure substances. To quantify the metabolites, a factor was calculated between a peak of the metabolite in a completely relaxed spectrum and the corresponding peak in a non-relaxed spectrum.

4.3.2.Total phenols and monomeric anthocyanins quantification

Phenols and anthocyanins were extracted into methanol as described (Boss et al. 1996) with modifications. Briefly four replicates of 10 frozen berries were carefully selected and peeled. Skins were ground in liquid nitrogen with a mortar and pestle. Methanol was added (3mL/gFW), and compounds extracted overnight at -20°C. Samples were then centrifuged at 4°C during 15 min at 16,100 x *g*. The supernatant was collected and two additional extractions (60 min each) were performed. The supernatants were mixed and filtered through 0.45 µm pore filters.

Total phenolic concentration was estimated using the Folin-Ciocalteu assay (Singleton et al. 1965) following the procedure described (Andre et al. 2007). Absorption at 755 nm was measured using a Power wave XS spectrophotometer (Biotek). A calibration curve was made with a gallic acid (GAE) solution and total phenolic contents were expressed as milligram of GAE per gram of fresh weight (FW). Total monomeric anthocyanins quantification was assessed by the pH-differential method according to Giusti and Wrolstad (2001) modified method. Briefly two dilutions of the sample, one with potassium chloride buffer (pH 1.0) and the other with sodium acetate buffer (pH 4.5) were made. After equilibrating the dilutions for 15 min, absorbance readings were made against water blank and results were expressed as mg malvidin equivalent per gram of FW.

4.4. Protein extraction

The mesocarp (flesh) and the exocarp (skin) of the berries were separated and the exocarp was ground in liquid nitrogen, in the presence of 10% (w/v) PVPP, with mortar and pestle. The powder was stored at -80°C prior to protein extraction. Three biological replicates composed by 10-20 berries were used for protein extraction.

Total protein was precipitated from 0.5 g of ground material with 3 volumes of cold 10% (w/v) TCA-acetone containing 60mM DTT, for 1 hour at -20°C. The protein samples were centrifuged at 10485 x *g* for 15 min at 4°C. The protein pellet was washed twice with cold acetone containing 60 mM DTT solution. Proteins were solubilized in 1mL SDS buffer (2% (w/v) SDS, 40 mM Tris base, 60 mM DTT), overnight at 4°C. The samples were centrifuged at 10485 x *g* for 15 min at 4°C. Removal of IEF interfering substances such as SDS was done with the 2D Clean-Up Kit (GE Healthcare), according to the manufacturer instructions. The pellet was then vacuum-dried for 5 min and solubilized in IEF solubilization buffer (2M thiourea, 7M urea, 0.4% (v/v) Triton X-100, 4% (w/v) CHAPS, 1% (v/v) IPG buffer 3-10 NL and 100 mM DTT) for 2 hours at 25ºC and constant agitation. Finally, the samples were centrifuged for 15 min at 16110 x *g* at room temperature. The protein concentration was determined using the 2D Quant Kit (GE Healthcare) with BSA as standard.

4.5. 2-DE

Proteins were IEF separated using IPGphor system (GE Healthcare) and Immobiline™ Drystrip gels (13 cm) with 3-10 non-linear pH gradients (GE Healthcare) at 20ºC. 250μg of protein were loaded per IPGstrip. The IPGstrips were rehydrated for 12 hours at 30V and IEF was conducted as followed: 100V (150 Vh), 250V (250 Vh), 1000V (1500 Vh), 2500 V (2500 Vh), 8000V (30min – gradient step) and 8000V (32000 Vh). After focusing, the gels were equilibrated for 2x15 min in 0.05 M Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS. DTT at 1% (w/v) was added to the first equilibration step and 2.5% (w/v) iodoacetamide to the second one. SDS-PAGE was performed on 12.5% acrylamide gels (Laemmli buffer system). The 2-DE gels were colloidal Coomassie blue G-250 stained according to (Neuhoff et al. 1988). Biological replicates were run in triplicate.

4.6. Image acquisition and analysis

2-DE gels were scanned using Image Scanner II (Amersham Biosciences). After imaging, the gels were analyzed by ImageMaster 2D Platinum software v5.0 (Amersham Biosciences). Spot detection and matching were 0. Only protein spots present in at least

two replicates and whose normalized volume (%) was >0.05 were selected and manually excised from the gels and stored at -80°C until MS analysis. The molecular weight of the proteins was predicted by the migration of 1-DE standards proteins (GE Healthcare) whereas the pI was calculated from the calibration curve (3-10NL IPG strips, GE Healthcare).

4.7. In gel digestion and protein identification by MS/MS

Excised protein spots were washed twice for 4h with 50% (v/v) ACN, 0.1M NH_4HCO_3, followed by a second wash for 2h. Prior to digestion the spots were reduced (10 mM DTT for 2 h at 37ºC) and alkylated (50 mM iodoacetamide for 15 min at room temperature) in the dark. The digestion was performed overnight with 0.125µg of trypsin (Promega) in 0.05M NH_4HCO_3 at 37ºC. The peptides in the supernatant were collected and the gel pieces were extracted with 0.1% (v/v) acetic acid/50% (v/v) ACN. The pool of extract and tryptic peptides was then dried in a speed-vac and re-dissolved in 0.1% (v/v) acetic acid/2% (v/v) ACN.

The trypsin digested proteins were analysed by capillary liquid chromatography tandem MS (LC/MS/MS) as described (Gatlin et al. 2000). The MS/MS spectra were searched against the 12x *Vitis vinifera* protein sequences (IGGP 12x; http://www.gramene.org/info/data/ftp/index.html, downloaded December 2010) using TurboSequest software (Gatlin et al. 2000). The MS/MS spectra were also searched against NCBInr, nonredundant database when no hit was obtained with 12x *Vitis vinifera* protein sequences. The databank was searched with Bioworks version 3.3.1. SP1 by setting the precursor ion tolerance to 1.4 Da, while the fragment ion tolerance was set to 1.0 Da when digests had been measured on an LCQ instrument, or 10 ppm precursor tolerance and 0.5 Da fragment ion tolerance for measurements on an LTQ Orbitrap instrument. Cleavage rules were set to fully enzymatic with cleavage at both ends, allowing 2 missed cleavages. Post filtering was set to the following parameters: ΔCN, 0.1; Xcorr versus charge state was 1.50 (1+), 2.00 (2+), 2.50 (3+); peptide probability, 0.5; protein probability 0.01 and minimally two peptides required for identification. False

positive discovery rate was estimated by searching the reversed 12x *Vitis vinifera* protein sequences. The Bioworks search results were exported to Excel files.

4.8. Statistical analysis

The statistical significance of changes in spot volumes was tested by the analysis of variance (ANOVA) using SPSS v12.0 software. Only spots that were differentially expressed at significant level ($p \leq 0.05$) were selected for further analysis. Genesis hierarchical clustering software was used to select the main classes of variations as described (Eisen et al. 1998; Sturn et al. 2002). The differences between the treatments in the metabolite data was also tested by ANOVA.

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6. LIST OF SUPPLEMENTARY MATERIAL

Supporting Information S1 Expression profile of exocarp proteins during berry development and ripening (44, 65, 78 and 98 days after flowering) and under three irrigation treatments (FI, RDI and NI). Proteins were grouped according to their functional classification (Table 1). Symbols represent means ± SE.
Supporting Information S2 MS/MS information of all the identified proteins.

Supplementary material is provided in the enclosed CD.

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Chapter IV

An ATP-binding cassette protein from grape berry (VvABCC1) transports anthocyanins and IAA-aspartate conjugates

Version of the manuscript entitled: *An ATP-binding cassette protein from grape berry (VvABCC1) transports anthocyanins and IAA-aspartate conjugates*

Francisco R[1]*, Nagy R[2]*, Regalado A[1], Sakakibara H[3], Chaves MM[1], Martinoia E[2]

[1]Instituto de Tecnologia Química e Biológica, Portugal; [2]Institute of Plant Biology, University Zürich, Switzerland; [3]RIKEN Plant Science Center, Yokohama, Japan.

* authors equally contributed to this work

Under revision to be submitted

R Francisco declares that have actively contributed to grape sampling, all the experimental work performed, data interpretation and manuscript writing.

ABSTRACT

The onset of grape berry ripening (*véraison*) is characterized by several events such as the accumulation of anthocyanins in the skin of red cultivars. In spite of the solid knowledge on anthocyanin biosynthesis and regulation, the participation of ATP-binding cassette proteins as vacuolar anthocyanin transporters has been matter of a long debate. Here, we report the identification of an ABC protein from grape berry (VvABCC1) able to transport *in vitro* either glucosylated anthocyanins as well as IAA-Asp conjugates. *VvABCC1* is expressed in the skin throughout berry ripening while in the mesocarp it is expressed only up to *véraison*. The transport of glucosylated anthocyanins is ATP, GSH and time-dependent, while the transport of IAA-Asp does not require GSH. Here we provide the first biochemical evidence for the involvement of an ABC protein from the ABCC subfamily in the vacuolar sequestration of both anthocyanins and IAA-amino acid conjugates supporting its role in the grape berry ripening process.

Keywords: grape berries, anthocyanins, auxin conjugates, ATP-binding cassette proteins, vacuolar sequestration

1. INTRODUCTION

Grape berries (*Vitis vinifera*) exhibit a double-sigmoid pattern of development with two distinct phases of growth separated by a *lag* phase (Coombe 1992). The onset of ripening (termed '*véraison*' by French viticulturists) occurs at the end of the *lag* phase and is characterized by glucose and fructose accumulation, metabolization of malate as the major carbon source for respiration, synthesis of compounds contributing to flavour and accumulation of anthocyanins in the skin of red cultivars (Kanellis and Roubellakis-Angelakis, 1993).

Fruits are classified either as climacteric or non-climacteric, based on physiological differences on their ripening hormonal pattern. Climacteric fruits such as tomato (*Solanum lycopersicum*), apple (*Malus domestica*), and banana (*Musa* spp) undergo a typical peak in ethylene synthesis and respiratory activity at the onset of ripening. In contrast, non-climacteric fruits such as strawberry (*Fragaria x ananassa*), and grape berries do not exhibit such a peak in ethylene concentration or respiration activity, and still little is known about its hormonal control of ripening (Tucker 1993; Adams-Phillips et al. 2004). Contrarily to early data reporting no significant changes in ethylene amount at the onset of ripening (Coombe and Hale 1973), recent experimental data reported the transient increase in ethylene prior to *véraison* suggesting its involvement in grape berry maturation (Chervin et al. 2004). Increased expression of ethylene receptors during the ripening of strawberries reinforces a putative role of this hormone in the ripening process of non-climacteric fruits (Trainotti et al. 2005). Besides ethylene, other hormones such as abscisic acid (ABA) (Davies et al. 1997; Gambetta et al. 2010; Koyama et al. 2010), brassinosteroids (Symons et al. 2006) and auxins (Davies et al. 1997) seem to be involved in the ripening process of grape berries.

The application of natural or synthetic auxins to berries, before the onset of ripening, has been shown to delay ripening processes such as softening, the accumulation of sugars and anthocyanins (Ban et al. 2003; Davies et al. 1997; Jeong et al. 2004). The molecular basis of the inhibition of anthocyanin accumulation following auxin treatment can be explained by the observed down regulation of genes involved in anthocyanin

synthesis (Ban et al. 2003; Davies et al. 1997; Griesser et al. 2008) as well as of the transcription factor *VvmybA1* (Jeong et al. 2004). The mechanisms underlying auxin regulation during fruit maturation are still elusive. However, the recent identification of an IAA-amino acid synthetase gene (*GH3*) from grape berries and the observed increase of the respective expression at the onset of ripening points out to its role in the establishment and maintenance of low IAA concentrations in ripening berries (Böttcher et al. 2010). According to the authors, the synthesis of these conjugates at, and after, the onset of ripening can represent a possible mechanism for the maintenance of low auxin in ripening fruits. ABC transporters have been suggested to be involved in auxin transport across the plasma membrane (Noh et al. 2001). Despite the hypothetical involvement of AtMRP5 in the transport of auxin conjugates to the vacuole (Gaedeke et al. 2001) up to now no biochemical evidence has been reported for the ABC-mediated transport of auxins to the vacuole.

Anthocyanins accumulated in the skin of red grapes are stored in the vacuole as 3-monoglucosydes of five basic anthocyanidins: cyanidin, delphinidin, peonidin, petunidin, and malvidin. The five basic anthocyanidins exist in hundreds of forms, possibly glucosylated and acylated at distinct sites however 3-*O*-glucosides are by far the most common derivates within *V. vinifera* species (Mazzuca et al. 2004). Anthocyanins are synthesized via the flavonoid pathway (Winkel-Shirley, 2001) in grapevine cultivars harboring the transcription factor *VvmybA1* gene for *UFGT* expression (Kobayashi et al. 2002, 2004). In spite of the solid knowledge on anthocyanin biosynthesis and regulation, only recently the mechanisms for anthocyanin transport into the vacuole started to be elucidated with the molecular cloning of the cDNAs encoding two *V. vinifera* Multidrug and Toxic Extrusion (MATE) proteins (Gomez et al. 2009). These proteins were shown to be primarily localized to the tonoplast and *in vitro* to transport acylated anthocyanins, which represent minor anthocyanins in grapevine (Gomez et al. 2009). Genetic evidence for the involvement of this class of transporters had been previously obtained for other flavonoids (Debeaujon et al. 2001). There has been a long debate as to whether ABC transporters are involved in anthocyanin transport to the vacuole (Klein et al. 2006;

Martinoia et al. 2007; Shiratake et al. 2007). A Multidrug Resistance-Associated (MRP) protein, an ABCC subfamily member of the ATP-binding Cassete superfamily (Verrier et al. 2008), from *Zea mays* (ZmMRP3) was localized in the tonoplast and antisense mutants of *ZmMRP3* were shown to exhibit a distinct pigmentation phenotype, as well as a significant reduction in anthocyanin content (Goodman et al. 2004). These data strongly suggest a role for ZmMRP3 in anthocyanin vacuolar compartmentation although no biochemical evidence had been given.

In order to answer the question whether ABC proteins can directly mediate anthocyanin transport, we decided to characterize grapevine VvABCC1, the closest homologue of ZmMRP3. Here we show that VvABCC1 can transport anthocyanins in the presence of glutathione as well as IAA-aspartate, an auxin conjugate produced during the ripening process. Therefore it is likely, that VvABCC1 is involved in grape berry maturation.

2. RESULTS

2.1. Phylogenetic analysis of ABCC-type proteins, expression and molecular cloning of VvABCC1 cDNA

V. vinifera ABCC protein sequences from the available grapevine database were chosen as candidates to carry out phylogenetic studies. The phylogenetic tree was created after the alignment of full-length amino acid sequences of 22 grapevine ABCC proteins, ZmMRP3 (Goodman et al. 2004) and all the Arabidopsis thaliana ABCC proteins present in Aramemnon database (Figure 1). From this tree it can be deduced that Vv06491001 (VvABCC1) and Vv06470001 exhibit the highest protein sequence similarity to ZmMRP3.

To know in which developmental stages and in which tissue VvABCC1 is expressed we performed a quantitative PCR analysis from day 49 to day 97 after flowering in the exocarp (skin) and mesocarp (pulp). In the exocarp VvABCC1 was found to be expressed throughout berry development with no significant alterations on gene expression, while in the mesocarp VvABCC1 was detected only until *véraison*. Before *véraison*, a higher expression of VvABCC1 was detected in mesocarp than in the exocarp (Figure 2).

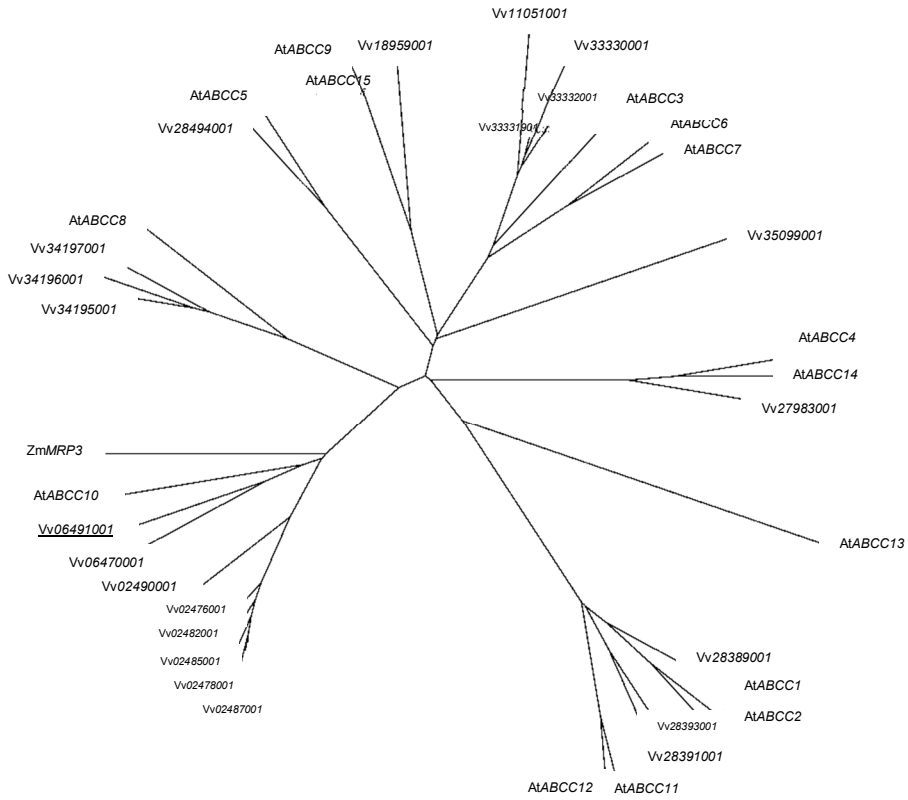

Figure 1 Phylogenetic comparison of representative members of the ABCC transporter family from Vitis vinifera, Arabidopsis thaliana and Zea mays (ZmMRP3). The unrooted phylogenetic tree shown is based on a multiple alignment of 38 full-length polypeptide sequences of ABC transporters produced by the CLUSTAL Program. The two letters preceding the protein names describe the organisms from which the sequences were derived: At, Arabidopsis thaliana; Vv, Vitis vinifera and Zm, Zea mays. Vv06491001 (VvABCC1) is underlined.

From berries collected at the *véraison* stage, the full-length cDNA encoding VvABCC1 (Vv06491001) was amplified by reverse transcription-PCR, cloned into the NotI restriction site of the plasmid pNEV-Ura (Sauer and Stolz, 1994) and fully sequenced. VvABCC1 encodes a 1480 amino acid residues protein with a predicted molecular mass of about 165 kDa and a calculated pI value of 6.38 (Compute pI/MW tool at http://www.expasy.ch/tools/pi_tool.html) (Figure1S; supplement data). All attempts to amplify the cDNA encoding Vv06470001, the closest VvABCC1 homologue, were

unsuccessful suggesting that its encoding gene is not expressed at detectable amounts in exocarp tissues at this grape berry developmental stage.

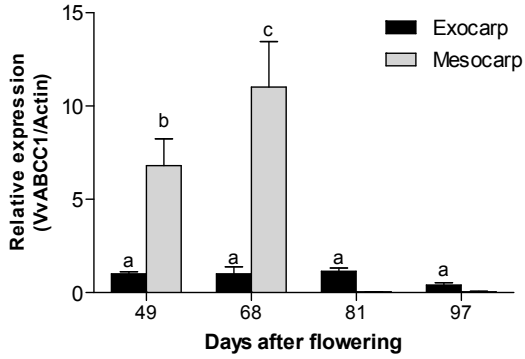

Figure 2 Quantitative real-time PCR expression profiling of *VvABCC1* in grape berry tissues (exocarp and mesocarp) during fruit maturation. Transcript levels are shown as fold change relative to VvABCC1 skin expression (49DAF). Gene expression was normalized using grapevine actin (GU585869) as a reference gene. Results are mean ± SE of three replicates.

2.2. VvABCC1 mediates malvidin-3-O-glucoside transport

In order to evaluate whether VvABCC1 is an anthocyanin transporter, the initial studies were conducted with malvidin-3-*O*-glucoside, the major anthocyanin of grape berry skins. VvABCC1 (pNEV-ABCC1) was expressed in yeasts defective in the YBT1 ABCC transporter (*ybt1* strain). Total microsomal membrane vesicles were isolated from the transgenic yeast. To assess the physiological intactness of the isolated vesicles an MgATP-dependent ΔpH was generated and monitored through the change in fluorescence emission of 9-amino-6-chloro-2-methoxyacridin (ACMA). ATP promoted a quenching of ACMA fluorescence and it was collapsed by the addition of $(NH_4)_2SO_4$, indicating that a proton gradient was formed across the membranes (data not shown).

After the transport experiments that were performed using the rapid filtration technique (Tommasini et al. 1996), quantification of anthocyanins taken up into the vesicles was carried out using HPLC. HPLC analysis showed distinct peaks for the two

anthocyanin monoglucosides malvidin-3-O-glucoside (M3G), delphinidin-3-O-glucoside (D3G) and the aglycon delphinidin (D) (Figure 3A).

M3G uptake into yeast microsomes could not be observed in the absence as well as in the presence of ATP. Since ABCCs have been shown to be mainly transporters for organic anions and that for some animal transporters glutathione was shown to be co-transported with substrates, we included the negatively charged glutathione (GSH) to our transport assays. Under this condition, M3G was taken up in a time-dependent manner by the yeast vesicles expressing VvABCC1. Values calculated from the HPLC analysis of vesicles incubated for 15 min clearly reveal that a relevant uptake of M3G was only observed when ATP and GSH were present (Figure 3B, C, D).

To test whether the transport is strictly ABCC-type protein driven, we included inhibitors known to inhibit ABC-type transport processes. In the presence of the sulphonylurea glibenclamide and the anion transport inhibitor probenecid, M3G transport was strongly reduced to 1 % and 3% respectively of the transport activity observed in the presence of ATP and GSH (Figure 3E).

Vanadate completely abolished M3G transport, and a similar inhibition was also observed by replacing ATP with the nonhydrolyzable ATP analogue AMPPNP (Figure 3E). These results further confirm that VvABCC1-mediated M3G transport is ATP-dependent and fulfils the pharmacology described for ABC transporters. No M3G transport was detected when experiments were performed on ice. These data is a further proof that the transport data presented in Figure 3B, C and D is not due to the non-specific permeability of yeast microsomes, or to the binding of M3G to yeast vesicles.

2.3. *VvABCC1 transports other 3-O-glucoside anthocyanidins*

To verify substrate specificity of VvABCC1, transport experiments were carried out with delphinidin-3-O-glucoside (D3G) and delphinidin (D) as substrates. No uptake was observed when the aglycone delphinidin was added as substrate (Figure 4A). VvABCC1 transported the 3-*O*-glucoside forms of both malvidin (M3G) and delphinidin (D3G), but

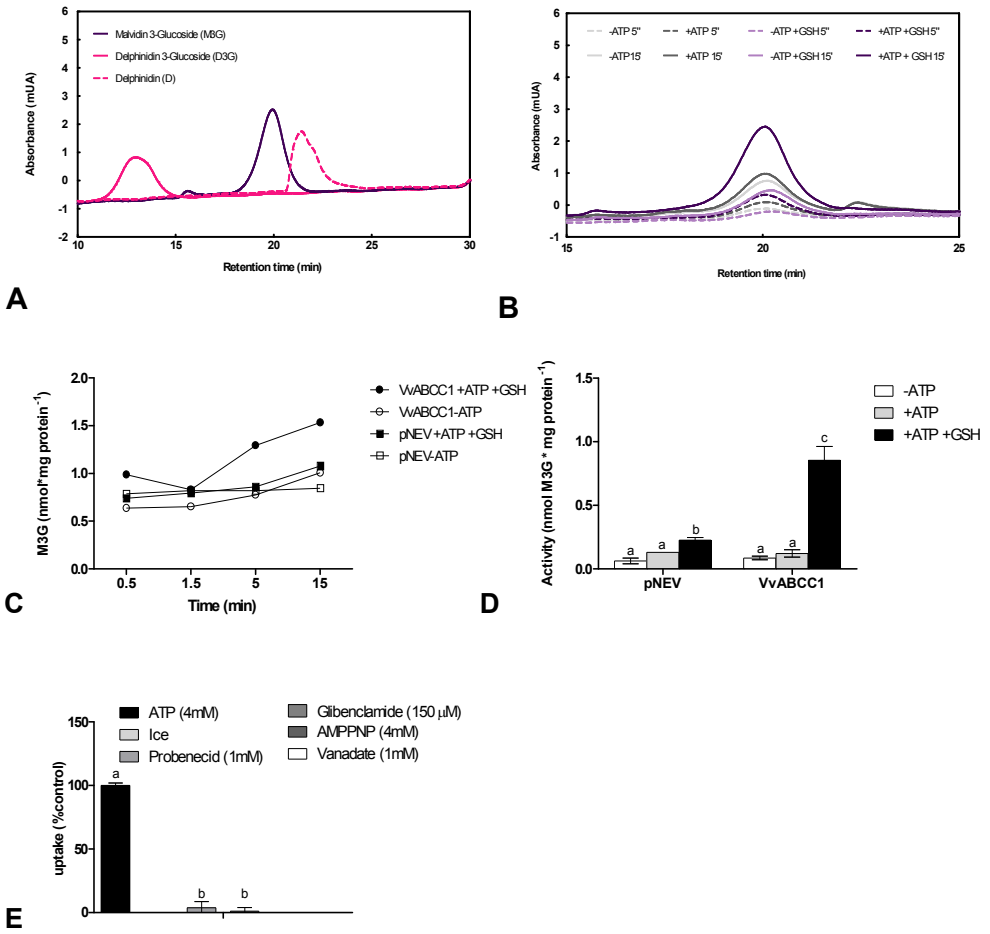

Figure 3 VvABCC1-mediated transport of malvidin-3glucoside (M3G) in yeast microsomal vesicles. A) HPLC profile of M3G, D3G, D standards; **B)** HPLC analysis of M3G uptake into yeast microsomal vesicles (pNEV) transformed with VvABCC1 in the absence of MgATP after 5 s (light grey dotted line) and after 15 min of incubation (light grey solid line), in the presence of MgATP after 5 s (dark grey dotted line) and after 15 min (light grey solid line) of incubation, in the absence of MgATP but in the presence of GSH after 5 s (light purple dotted line) and after 15 min (light purple solid line) of incubation and finally in the presence of both MgATP and GSH after 5 s (dark purple dotted line) and after 15 min (dark purple solid line) of incubation; **C)** Time dependent-uptake of M3G into vesicles isolated from yeasts transformed either with VvABCC1 or the empty vector (pNEV); **D)** Quantification of M3G uptake by VvABCC1 and the empty vector; **E)** Inhibition of M3G uptake by ice, probenecid, glibenclamide, vanadate and the non hydrolyzable ATP analogue (AMPPNP). Results are mean ± SE of three independent uptake experiments from three independent microsomes preparations. Ice, AMPPNP and vanadate values were zero. Different letters indicate significance differences between conditions ($p \leq 0.01$).

the transport was more efficient for M3G than for D3G (Figure 4A). Similarly to M3G, D3G uptake was observed only in the presence of both MgATP and GSH (Figure 4B).

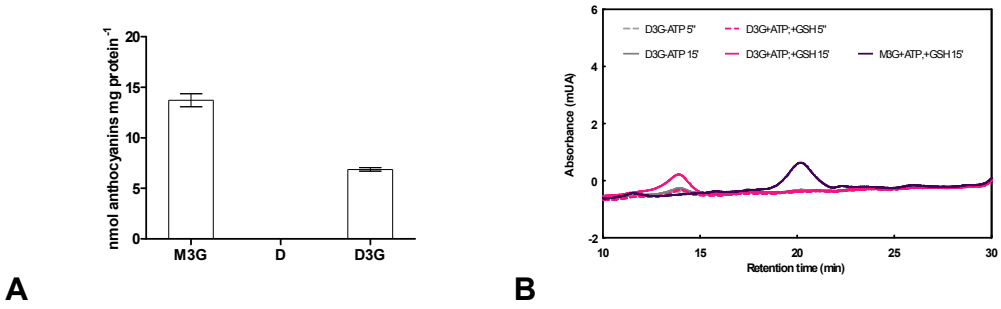

Figure 4 VvABCC1-mediated transport of delphinidin 3-glucoside (D3G), delphinidin (D) and malvidin 3-glucoside (M3G) in yeast microsomal vesicles. **A)** Quantification of MgATP/GHS uptake activity of M3G, D3G and D by VvABCC1; **B)** HPLC analysis of D3G uptake into VvABCC1-transformed yeasts in the absence of MgATP after 5 s (light grey dotted line) and after 15 min of incubation (light grey solid line), in the presence of both MgATP and GSH after 5 s (pink dotted line) and after 15 min (pink solid line) of incubation; M3G uptake in the presence of both MgATP and GSH after 15 min of incubation (dark purple solid line); no D absorbance peak was observed. Results are mean ± SE of two independent uptake experiments from two independent microsomes preparations.

2.4. VvABCC1 mediates IAA-aspartate transport

As stated in the introduction, grape berry maturation is characterized by a decrease in free auxin and an increase in auxin conjugates. This was also true in our case, where a strong decrease of IAA at *véraison* could be observe followed by a steadily increase of IAA-Asp after the onset of *véraison* (Figure 5). In contrast, IAA-Ala, IAA-Ile, and IAA-Leu could not be detected. Since IAA-Asp is an organic anion, hence a potential substrate for ABCC transporters and since it is known that ABC proteins can transport many unrelated substrates (Rea 2007), it was hypothesised whether VvABCC1 could also act as an IAA-Asp transporter. Uptake experiments using ^{3}H-IAA-Asp were carried out with yeast

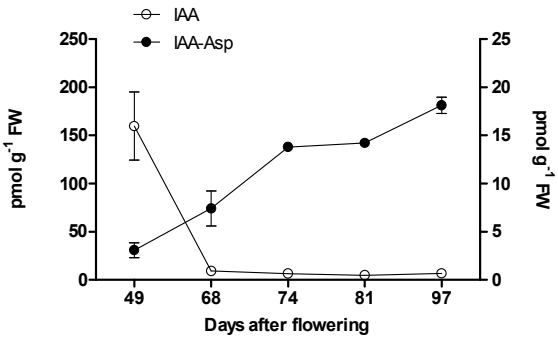

Figure 5 IAA and IAA-Asp concentration in developing grape berries quantified by LC-MS. Results represent mean ± SE of four replicates

vesicles prepared from *ybt1* cells transformed with either the empty vector (pNEV) or pNEV-VvABCC1. Indeed, VvABCC1-dependent IAA-Asp conjugate uptake could be observed. However, in contrast to M3G and D3G, VvABCC1-mediated IAA-Asp transport did not depend on the presence of GSH (Figure 6A). Microsomes isolated from yeast cells carrying the empty vector showed a low level of ATP-dependent IAA-Asp uptake (Figure 6B). The residual transport in the absence of VvABCC1 might be due to one of the ABCC genes still present in *ybt1*. Inhibition assays (Figure 6C) showed that in the presence of glibenclamide and probenecid the transport of IAA-Asp was strongly inhibited. AMPPNP reduced the IAA-asp transport to 8% and vanadate, an efficient inhibitor for most ABC-type protein-mediated transport, had only a partial inhibitory effect (40%). An 80% inhibition of the transport activity was observed when performing experiments on ice.

3. DISCUSSION

3.1. VvABCC1 transports glucosylated anthocyanins

Transport of anthocyanins across the tonoplast has been matter of a long debate. Two major mechanisms have been proposed: a primary transport, mediated by ATP-binding

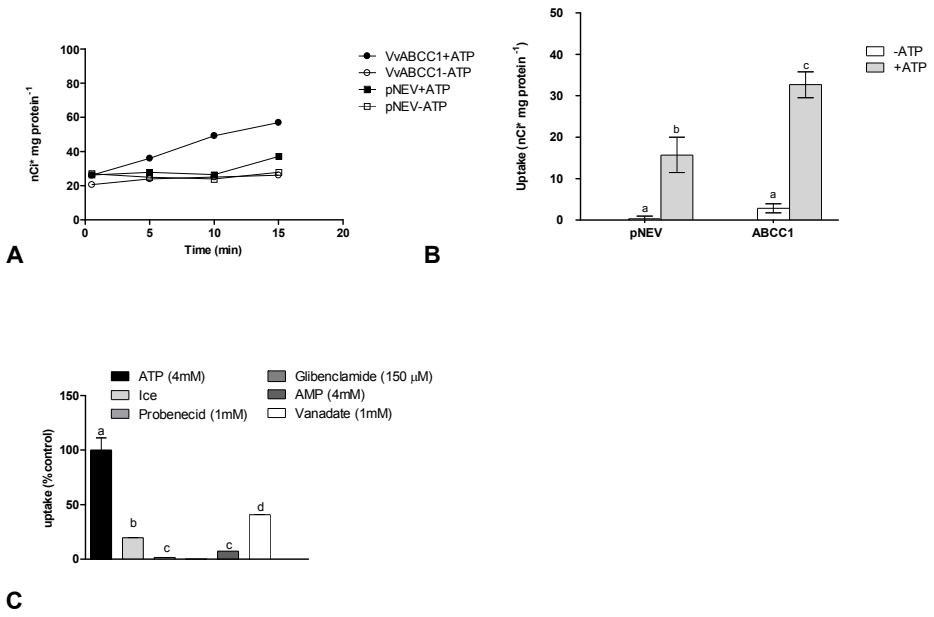

Figure 6 VvABCC1-mediated transport of indole-3-acetic acid-aspartic acid (IAA-Asp) in yeast microsomal vesicles. VvABCC1-mediated transport of indole-3-acetic acid-aspartic acid (IAA-Asp) in yeast microsomal vesicles. **A**) Time dependent-uptake of IAA-Asp into vesicles isolated from yeasts transformed either with VvABCC1 or the empty vector (pNEV); **B**) Quantification of IAA-Asp uptake by VvABCC1 and the empty vector; **C**) Inhibition of IAA-Asp uptake by ice, probenecid, glibenclamide, vanadate and the non hydrolyzable ATP analogue (AMPPNP). Results are mean ± SE of three independent uptake experiments from three independent microsomes preparations. Glibenclamide values were zero. Different letters indicate significance differences between conditions ($p \leq 0.01$).

cassette (ABC) transporters and a secondary activated transport, mediated by MATE proteins (Grotewold, Davies, 2008). Only recently the mechanism for anthocyanin transport into the vacuole started to be elucidated with the molecular cloning of cDNAs encoding two V. vinifera MATE proteins (Gomez et al. 2009). These proteins were shown to be primarily localized to the tonoplast and to transport anthocyanin-acylglucosides in vitro (Gomez et al. 2009). This suggests that another mechanism should be involved in the transport of the predominant nonacylated grape berry anthocyanins. Our findings

prove that, in vitro, VvABCC1 has the ability to transport glycosylated anthocyanins in an ATP and GSH time-dependent manner, suggesting that VvABBC1 is part of the mechanisms of vacuolar anthocyanins transport. Taken this and Gomez et al. (2009) results the hypothesis that different anthocyanin structures are transported by different mechanisms (Martinoia et al. 2007) is corroborated. For long that ABCC (previously named MRP proteins) have been suggested to be involved in the transport of anthocyanins (Marrs et al, 1995; Lu et al. 1997; Liu et al. 2001; Kitamura et al. 2004) being the studies of Goodman et al.(2004) the most compelling evidence of that. Goodman and co-workers studied a maize antisense mutant of a vacuolar ABCC protein (ZmMRP3), that exhibited a reduction in anthocyanin production and pigment mislocalization although no biochemical prove of the transport was presented. More recently, the ectopic expression of an anthocyanin transcription factor (VvMybA1) in grapevine hairy roots system (Cutanda-Perez et al. 2009) revealed the induction of several genes specifically related with the last steps of anthocyanins metabolism, namely a MATE encoding gene and in spite of to a lesser extent an ABCC protein (AtABCC10). To date, few members of the ABCC subfamily were functionally characterized (Rea 2007). The Arabidopsis ABCC members are the best characterized plant ABCC proteins. Five unique AtABCCs, AtABCC 1-5, have been cloned and shown to encode functional transporters after heterologous expression in S. cerevisiae (Rea 2007). Apart the transport of xenobiotics, heavy metals and herbicides, these proteins are also involved in the transport of flavonoids (Martinoia et al. 1993, 2002; Rea et al. 1998, Klein et al. 2000; Theodoulou, 2000). Here we present the first experimental biochemical evidence that an ABCC protein transports anthocyanins.

Grape berries accumulate several chemical derivatives of five anthocyanidins (malvidin, cyaniding, delphinidin, petunidin and peonidin). Our results show that VvABCC1 preferentially transports M3G when compared to D3G. One can hypothesize that proteins having closest homology to VvABCC1 could be involved in the transport of the different forms of anthocyanins present in grape berry. It is interesting to stress that in spite of the recognized broad range of ABCC substrates, VvABCC1 showed a certain

degree of specificity since it was not able to transport either leukotriene C4 or estradiol-17-β-D-glucuronide (data not shown).

In grape berry skin the expression of genes encoding enzymes involved in the anthocyanins biosynthetic pathway was detected up to 4 weeks after flowering, followed by a decrease and finally close to *véraison* a peak of expression is again observed (Boss et al. 1996). It was proposed that this pattern may be related with the overlapping activity of some of these enzymes in other branch pathways of flavonoids biosynthesis, namely proanthocyanidins (Boss et al. 1996). VvABCC1 was shown to be expressed in skin tissues throughout berry development and ripening, i.e. even before the onset of *véraison*, where no anthocyanins synthesis occurs. Although further work is needed to confirm the in vivo function of VvABCC1, it would be interesting to test whether this overlapping performance of certain enzymes could also be extended to VvABCC1 ability to transport other compounds, such as proanthocyanidins that accumulate in grape berries mostly during pre-*véraison*.

3.2. VvABCC1 transports glucosylated anthocyanins only in the presence of GSH

In the present study VvABCC1-mediated transport of M3G and D3G was shown to be possible only in the presence of GSH. Glutathionated compounds are high-affinity substrates for ABCCs from different species (Ishikawa et al. 1997; Rea et al. 1998). The close functional resemblance of plant ABCCs to the mammalian GS-conjugated Mg^{+}-ATPases (or GS-X pumps) was recognized through the uptake of two model GS-conjugates and the glutathionated herbicide metolachlor-GS by isolated vacuoles or membrane vesicles (Martinoia et al. 1993; Li et al. 1995). It had been known for some time that the bronze coloration of maize Bronze-2 (*bz2*) mutant is due to the cytosolic accumulation of oxidized and crosslinked anthocyanins. A rational basis for the *bz2* phenotype was obtained through the identification of *Bz2* as a GST encoding gene putatively capable of conjugating C3G with GSH (Marrs et al. 1995). Furthermore, Lu and co-workers (1997) provided evidence that *At*ABCC1 transports *in vitro* the synthesized C3G-GS at rates several fold higher than the model GS-conjugate DNP-GS, supporting glutathionation of anthocyanins as a pre-requisite for this type of transport. However, to

our knowledge the occurrence of naturally conjugates between anthocyanins and GSH has not been obtained so far. Considerable evidence pointed to the involvement of GSTs in the last steps of anthocyanins biosynthesis. Indeed, analysis of maize (*Bronze 2*), Petunia (*AN9*) and Arabidopsis (*TT19*) mutants led to the identification of genes encoding widely divergent GSTs able to functional complementation in anthocyanin sequestration (Alfenito et al. 1998; Kitamura et al. 2004). More recently, Conn et al. (2008) showed that two VvGSTs are able to complement the maize *bronze-2* phenotype however the interaction between anthocyanins and GST has not been clearly characterized. Whether GSTs act as a *ligandin* or through glutathionation is still a matter of debate. Taken together the available data and the presented results it can be concluded that glutathione is essential for VvABCC1-mediated glucosylated anthocyanins transport, although further studies are required to clarify this matter since neither anthocyanin glutathionation or glutathione S-transferase (GST) activity was undertaken in the present study.

3.3. VvABCC1 is involved in grape berry development and ripening

The expression of VvABCC1 in mesocarp tissues, where anthocyanins do not accumulate, suggests that this protein functions beyond anthocyanin transport. Our findings demonstrate that VvABCC1 is able to transport IAA-Asp conjugates in an ATP and time-dependent manner.

For long that phytohormones have been proposed as substrates for ABC proteins (Noh et al. 2001). Martinoia et al. (2002) suggested that it is likely that growth/developmental processes in plants can be modulated in several ways by ABC transporters. The molecular mechanisms of auxin action during fruit development are to a great extent still unclear. It has been reported that exogenous auxin can delay grape ripening but the role of endogenous auxin in ripening is still largely unknown (Davies and Böttcher, 2009). Microarray studies of grape berries revealed that auxins may have a role throughout berry development/ripening (Deluc et al. 2007) in berry pericarp (Grimplet et al. 2007). In grape berries it has been reported that IAA tends to increase to a maximum just after anthesis, declining thereafter (Cawthon and Morris, 1982). We

observed that free IAA in deseeded berries decreased until 68 DAF when it reached a residual concentration that was maintained throughout the final stages of berry ripening. Moreover, the decrease of free-IAA was accompanied by an IAA-Asp accumulation in a ripening-related manner. Recently, Böttcher et al. (2010) reported similar results and proposed that the formation of these conjugates is associated to the expression/activity of an indole-3-acetic acid-amido synthetase (GH3-1). Interestingly, IAA-Asp was the only IAA-conjugate detected in our study. Böttcher and co-authors (2010) suggested that IAA-Asp conjugation might represent the mechanism for the maintenance of low IAA during fruit ripening (Böttcher et al. 2010). In *Arabidopsis*, catabolic conjugation mechanisms are up-regulated in response to elevated IAA (Woodward and Bartel, 2005), being postulated that IAA-Asp is a candidate for detoxification of excess IAA (Ljung et al. 2002). The observed accumulation profile of IAA-Asp suggests that the formation of these compounds is related with the initiation of the ripening events. IAA-Asp conjugation is an irreversible process that leads to oxidation followed by IAA catabolism. In this context it is interesting to note that VvABCC1 was highly expressed up to *véraison* in mesocarp tissues. Additional studies are required to determine the biological relevance of these findings.

In conclusion, this study provides biochemical evidence of specific transport of glycosylated anthocyanins mediated by an ABCC transporter. Moreover, this transporter has also the ability to transport IAA-Asp conjugates. The *in vivo* characterization of this protein will provide further evidence about its role in the general mechanism of flavonoids vacuolar sequestration during fruit development and ripening.

4. MATERIALS AND METHODS

4.1. Plant Material

Berry samples (Vitis vinifera cv. Aragonez) were collected from an experimental plot at the commercial vineyard Monte dos Seis Reis (South of Portugal, Estremoz, Portugal). Berries were collected during the summer season of 2007 at five representative stages of development and maturation: pre-*véraison* at pea size (49DAF), *véraison* (68DAF)

where 50% berries were coloured, fully coloured berries (74DAF), maturation (81DAF) and fully-maturation (97DAF). Collected berries were immediately frozen in liquid nitrogen and stored at -80ºC before use.

4.2. Molecular cloning of VvABCC1

The full-length cDNA of *VvABCC1* was amplified using the high fidelity PCR Extender polymerase mix (5PRIME) according to the manufacturer's instructions. The following primers were used: VvABCC1 forward (5'-ATAAGAATGCGGCCGCATGGGGGATCTGTGGACTATGTTTTGTGGG-3') and VvABCC1 reverse (5'-ATAAGAATGCGGCCGCTCAATGTGATTCCGCTGAGTGAAAATGGGACC-3'), where the NotI restriction site is underlined. The resulting PCR product was cloned into the *Not*I restriction site of the yeast expression vector pNEV-Ura (Sauer and Stolz, 1994).

4.3. Functional analysis in yeast vesicles

The *ybt1* yeast mutants were transformed as described by Gietz and Woods (2002) either with pNEV-Ura harbouring no insert (pNEV) or with *VvABCC1* cDNA cloned into pNEV-Ura (pNEV-VvABCC1). Transformants were selected on minimal synthetic dropout medium lacking uracil. Yeast microsomes for *in vitro* transport studies were isolated as described by Tommasini et al. (1996) and intactness of the vesicle fractions was evaluated by quenching of the fluorescent dye 9-amino-6-chloro-2-methoxyacridin (ACMA) (Gomez et al. 2009). Briefly, vesicle extracts (300 µg) were added to a reaction solution (1 mL) containing 2µM ACMA, 0.4 M glycerol, 6 mM MgSO4, 1 mM dithiothreitol, 0.1M KCl, 20 mM Tris-MES, pH 7.4 and 5mM MgATP. The fluorescence intensities were measured by spectrofluorimetry and the ACMA signal was acquired at 485 nm after excitation at 425 nm. Evaluation of proton gradient dependent uptake of ACMA, into yeast vesicles was performed through addition of 25 mM $(NH4)_2SO4$ to the reaction solution.

The uptake experiments to study the transport of malvidin-3-*O*-glucoside (M3G), delphinidin-3-*O*-glucoside (D3G), delphinidin (D), indole-3-acetic acid conjugated with aspartate (IAA-Asp), into membrane microsomes were performed using the rapid

filtration technique (Tommasini et al. 1996) with nitrocellulose filters (0.45-mm pore size; Millipore)

Anthocyanin transport assays were performed with 0.2 mL of vesicles mixed to ice-cold transport buffer (0.4 M glycerol, 0.1 M KCl, 20 mM Tris-MES, pH 7.4) and freshly added 1 mM dithiothreitol, 6mM or 1mM $MgSO_4$ (if in the presence or absence of MgATP, respectively), 100µg/mL creatine kinase and 10 mM creatine-phosphate; 0.5 mM of M3G, D3G or D were assayed either in the presence or absence of 4 mM MgATP and 5 mM GSH in a total reaction volume of 0.8 mL. All steps were performed with samples kept on ice. At time points indicated, 0.35 mL of the reaction mixture were immediately loaded on a pre-wet filter and rapidly washed with 3x 2 mL of ice-cold transport buffer. The filter-bound anthocyanins were dissolved by adding 50% (v/v) methanol, 0.1% (v/v) HCl at 37ºC during 10 min. The eluted anthocyanins were then quantified by HPLC as described by Marinova et al. (2009). Briefly, HPLC separation was performed on a CC 250/4 Hypersil 100-5 C18HD column (Macherey-Nagel) with 0.6% perchloric acid (A) and methanol (B) at a total flow rate of 1mL/min in the following gradient: 0 to 20 min from 5 to 60% B over A. Parameters were controlled by a Gynkotek liquid chromatograph (Dionex) equipped with a UVD340S diode array detector set at 520 nm, 330nm, and 280 nm for anthocyanin identification. Transport of IAA-Asp conjugates were performed basically in the same way, using 0.1 mL of vesicles mixed to the transport buffer (total reaction volume of 0.65 mL) in the presence 0.1 µCi/filter, respectively. At time points indicated, 0.1 mL of the reaction mixture was loaded on a pre-wet filter and rapidly washed with 3x 2 mL of ice-cold transport buffer. Counts were detected by adding to the filters 3ml UltraGold scintillation cocktail. Measurements were done accordingly to established software. Inhibition assays were carried out in the presence of the following potential inhibitors: 4mM anyl-5'-yl imidodiphosphate (AMPPNP), 1mM probenicide, 150 µM glibenclamide and 1mM vanadate. For the inhibition experiments, yeast microsomes were incubated at room temperature in the reaction mix that contains the indicated potential inhibitors. After 10 minutes incubation, anthocyanins were added and the uptake experiment procedure was performed as described above. For the

experiments on ice, the tubes containing the reaction mix and M3G were placed on ice immediately after the addition of yeast microsomes.

The *ybt1* yeast mutant was used for the transport assays of anthocyanins and auxin-aspartate conjugate. All experiments were repeated three times with independent vesicle preparations unless stated otherwise.

4.4. Gene expression analysis of VvABCC1

Total RNA was extracted from frozen berry tissues (exocarp and mesocarp) according to Reid et al. (2006). Total RNA was purified using an RNeasy kit (Qiagen) with the addition of an on-column DNase I digestion. Total RNA (0.5 μg) was reverse transcribed using reverse transcriptase M-MULV (Roche Diagnostics) priming with oligo$(dt)_{18}$ according to the manufacturer's recommendations. Transcript levels were determined by qRT-PCR using iQ5 RT-PCR (Bio-Rad, Hercules, CA). Reactions were performed in a final reaction mixture of 20 μL of cDNA (diluted 1:10), 0.25μM gene-specific primers and master mix iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reaction conditions for the thermal cycling were as following: after enzyme activation at 95ºC for 3 min, amplification was carried out in a three-step PCR procedure with 40 cycles of 15 s at 95ºC for denaturation, 10 sec at 60ºC for annealing and 10 sec at 72ºC for extension. All reactions were performed in triplicate with three biological replicates. Gene primer sequences used in the qRT-PCR analyses were as follows: VvABCC1 forward, 5'-ATGGGGGACTGTGGACTATG -3' and VvABCC1 reverse 5'- CTGGACATGAACTGGCTTG-3'. Dissociation curves were analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 55ºC to 95ºC. Transcript levels were calculated using the standard curve method and normalized against the grapevine actin gene (GU585869) as described by Pfaffl (2001).

4.5. IAA and IAA-amino acid conjugates quantification

Frozen berries were deseeded and macerated to a fine powder in liquid nitrogen. Methanol extraction was performed (1mL/gFW). Free IAA and IAA conjugates were determined as described (Kojima et al. 2009).

4.6. Phylogenetic analysis of ABCC proteins

Grapevine sequences were searched on *V. vinifera* genome database (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) and on plant membrane database (http://aramemnon.botanik.uni-koeln.de/), where Arabidopsis thaliana homologues were searched. Multiple sequence alignment and phylogenetic tree construction was performed with ClustalW (http://align.genome.jp/).

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Chapter V

Grapevine under deficit irrigation – hints from physiological and molecular data

Reformatted version of the article entitled: *Grapevine under deficit irrigation – hints from physiological and molecular data*

Maria Manuela Chaves[1,2], Olfa Zarrouk[2], Rita Francisco[2], Joaquim Miguel Costa[1,2], Tiago Santos[1,2] Ana Paula Regalado[2], Maria Lucília Rodrigues[1], Carlos Manuel Lopes[1]

[1]Instituto Superior de Agronomia, Portugal; [2]Instituto de Tecnologia Química e Biológica, Portugal

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R Francisco declares that actively contributed to this review with the proteomic and metabolite data presented in Figure 3, and as well in the manuscript writing.

ABSTRACT

A large proportion of vineyards are located in regions with seasonal drought (e.g. Mediterranean-type climates) where soil and atmospheric water deficits, together with high temperatures, exert large constraints on yield and quality. The increasing demand for vineyard irrigation requires an improvement in the efficiency of water use. Deficit irrigation emerged as a potential strategy to allow crops to withstand mild water stress with little or no decreases of yield, and potentially a positive impact on fruit quality. Understanding the physiological and molecular bases of grapevine responses to mild to moderate water deficits is fundamental to optimize deficit irrigation management and identify the most suitable varieties to those conditions. How the whole plant acclimates to water scarcity and how short and long distance chemical and hydraulic signals intervene are reviewed. Chemical compounds synthesized in drying roots were shown to act as long-distance signals inducing leaf stomatal closure and/or restricting leaf growth. This explains why some plants endure soil drying without significant changes in the shoot water status. The control of plant water potential by stomatal aperture via feed-forward mechanisms is associated with the 'isohydric' behaviour in contrast with 'anysohydric' behaviour where lower plant water potentials are attained. We discuss differences in this respect between grapevines varieties and experimental conditions. Mild water deficits also exert direct and/or indirect (via the light environment around grape clusters) effects on berry development and composition; a higher content of skin-based constituents (e.g. tannins and anthocyanins) has generally being reported. Regulation under water deficit of genes and proteins of the various metabolic pathways responsible for berry composition and therefore wine quality are reviewed.

Keywords: *Vitis vinifera*, varieties; stomatal conductance (g_s); intrinsic water-use-efficiency (WUEi, An/g_s); isohydric; anisohydric; abscisic acid; berry composition

1. VINEYARDS AND WATER SCARCITY

Most of the world's wine-producing regions experience seasonal drought. With an increase in aridity predicted in the near future according to global climate models (IPCC 2007), water deficits may become a limiting factor in wine production and quality. Global warming is also affecting grapevine development as indicated by changes in phenology, and earlier harvests observed throughout the world (Jones and Davies 2000, Webb et al. 2007), with some European regions coming closer to the thresholds of temperature and rainfall for optimum grapevine growth (Jones et al. 2005). In recent years, water deficit is also occurring in cool climate wine regions that exhibit special topography (van Leeuwen and Seguin, 2006; Zsófi et al. 2009). The frequency of extreme events like heat waves or heavy rains is also predicted to increase, with very negative effects on yield and quality of grapes. Sudden supra-optimal temperatures under conditions of water scarcity may lead to massive leaf shedding, with a consequent source-sink imbalance and incomplete berry maturation due to insufficient available carbohydrates. These effects are unlikely to be uniform across varieties (Jones et al. 2005, Schultz, 2000). The constraints posed by climate change require adaptive management, namely irrigation to stabilise yield, maintaining or improving wine quality (Dry and Loveys 1998; Medrano et al. 2003; Chaves et al. 2007) and other associated management techniques (for example, soil cover) to minimize the effects of concentrated rainfall (Schultz 2007; Monteiro and Lopes 2007). The search for varieties adapted to growing seasons with altered length and displaying higher resilience to environmental stress is also critical to optimum berry ripening.

On the other hand, the enhanced pressure on water resources increased the global perception of the need to reduce the 'water footprint' for irrigated crops (www.fao.org/nr/water/aquastat/data/query/index.html) (Cominelli et al. 2009). An improvement in the productivity of water use is therefore required in vineyard management, with finely-tuned deficit irrigation being able to fulfil that role.

To understand the physiological and molecular bases of plant responses to mild to moderate water deficits is therefore of utmost importance to modulate the appropriate

balance between vegetative and reproductive development, to improve crop water-use (Blum 2009) and to control fruit quality under deficit irrigation (Chaves et al. 2007). Chemical signals are important players in plant adaptation to environmental stresses. Since the mid-1980s evidence was provided on the signalling role of compounds synthesized in drying roots of different species (including grapevines); they have associated with leaf stomatal closure and/or inhibition of meristematic development (Loveys 1984; Davies and Zhang 1991). Although root-sourced chemical signalling is widely accepted, the identity and regulation of these signals is still under debate (Holbrook et al. 2002; Schachtmann and Goodger, 2008). Nevertheless, such knowledge enabled us to manipulate responses to soil water availability in some crops, so that changes in shoot water status are minimized and the performance under moderate stress is improved (Davies et al. 2002; Chaves and Oliveira, 2004).

The timing and intensity of water deficits influence, the extent of alterations occurring in berry metabolism and therefore in wine colour and flavour (namely the aroma). Whether these effects are acting predominantly through berry size or the synthesis of berry compounds will also be discussed. The accumulated knowledge made possible by studies of transcriptomics and proteomics during different stages of berry development in different varieties and environmental conditions will also be highlighted.

2. THE RATIONALE FOR DEFICIT IRRIGATION - WHY MILD TO MODERATE WATER DEFICIT MAY BE FAVOURABLE TO GRAPE BERRY QUALITY

Grapevines are well-adapted to semi-arid climate like the Mediterranean, due to the large and deep root system and physiological drought avoidance mechanisms, such as an efficient stomatal control of transpiration and of xylem embolism (Lovisolo et al. 2002), and/or the ability to osmotically adjust (Rodrigues et al. 1993; Patakas and Noitsakis 1999). However, the combined effect of drought, high air temperature and high evaporative demand during summer in these areas is known to limit grapevine yield and berry and wine quality (Escalona et al. 1999; Chaves et al. 2007; Costa et al. 2007). Dramatic reductions in plant carbon assimilation may occur due to severe decline in

photosynthesis under supra-optimal leaf temperatures combined with water deficits, as well as to a partial loss of canopy leaf area (Chaves et al. 2003, 2007; Flexas et al. 1998, 2002; Sousa et al. 2003, 2005b; Maroco et al. 2002; Santos et al. 2007). The use of irrigation in these environments arises as a solution to prevent excessive canopy temperature, keeping quality in wine production, and in more extreme cases, guarantee plant survival. Nevertheless, irrigation remains an object of large debate. On the one hand, small water supplements may increase yield and maintain or even improve berry quality (Matthews and Anderson 1989; Santos *et al.* 2003, 2005). On the other hand, irrigation may promote excessive vegetative growth with a negative impact on berry pigments (colour) and sugar content, and therefore decrease wine quality (Bravdo et al. 1985; Dokoozlian and Kliewer 1996). Larger canopy leaf area will also tend to increase the incidence of fungal diseases (Dry and Loveys 1998).

Modern irrigation management is shifting from an emphasis on production per unit soil area towards maximizing water productivity (production per unit of consumed water) (Fereres and Soriano 2007). Besides, we must consider not only the total seasonal water available in a region but also the timing when water deficits are likely to occur, in order to adjust water needs to the available resources, using a limited supply of water most effectively (Passioura 2007). The use of deficit irrigation strategies, implying that water is supplied at levels below full crop evapotranspiration (ETc) throughout the growing season or in specific phenological stages, rely on observations in several crops subjected to moderate water deficits that yield is not significantly reduced and quality of production may even increase under such conditions. This has been the case for several fruit tree crops (see review by Fereres and Soriano 2007) and grapevines (Dry et al. 2001; Chaves et al 2007). In addition to the classic deficit irrigation (DI) that does not require specific technical control, two other deficit irrigation strategies - regulated deficit irrigation (RDI) and partial rootzone drying (PRD) – have been applied in recent years by finely tuning deficit irrigation respectively, in the scales of time (specific timing of the application) and space (alternating dry-wet zones). Although deficit irrigation is already applied to vast regions all over the world in a more or less

uncontrolled/unsophisticated way, the scientific knowledge underlying its optimal functioning is still needed.

Under RDI plant water status is maintained within pre-defined limits of deficit (with respect to maximum water potential) during certain phases of the seasonal development, normally when fruit growth is least sensitive to water reductions (Kang and Zhang 2004). The rational underlying this practice is that optimization of numbers of fruits, fruit size and quality will be achieved by keeping grapevine vigour in balance with potential production. If water deficit is applied early in the season the effects will be achieved mostly through a reduction of berry cell division (McCarthy et al. 2002); if water deficits are imposed at later stages, then the major effect will be an inhibition of berry growth (Williams and Mathews 1990).

In PRD, roots are exposed to alternate drying and wetting cycles. Theoretically, roots of the watered side of soil will maintain favourable plant water relations, while dehydration in the other side will induce chemical signalling that will reach the leaves via the transpiration stream, reducing stomatal conductance and/or growth (Davies et al. 1994; Santos et al. 2003; Kang and Zhang 2004; Costa et al. 2007). This will bring about an increase in water-use efficiency (WUE). PRD irrigation may also have an impact on root growth, leading to increased root development in the deeper soil layers as shown by Dry et al. (2000) and Santos et al. (2007). Moreover, an increase in root hydraulic conductance, putatively resulting from aquaporin stimulation by ABA, and the induction of new secondary roots was reported in fruit trees subjected to PRD (Kang and Zhang 2004).

There are however contrasting results in the literature, several studies in grapevine reporting no significant differences between PRD and DI (Pudney and McCarthy 2004; Baeza and Lissarrague, 2005 Bravdo et al. 2004, Gu et al. 2004). These apparent contradictions may be related to differences in the intensity of the chemical signalling under PRD irrigation that seems to be dictated by the type of soil, the prevalent rainfall and evaporative demand in the region, as well as the frequency of switching irrigation from one side of the rootzone to the other (Dry et al. 2001; Chaves et al. 2007).

Genotypic differences in stomatal sensing of water deficits or the delivery of ABA by the root-stock, may also explain different results (Antolin et al. 2006; De la Hera et al. 2007). Drought sensitive varieties may respond better to PRD (Souza *et al.* 2005a). The type of soil will impact on the extent of soil water redistribution, which in turn will buffer dehydration in the dry rootzone. Bravdo (2005) suggests that hydraulic redistribution from deeper to shallower roots may prevent under field conditions the clear results obtained in potted plants subjected to PRD under split root systems (Davies et al. 2002). Dry (2005) also suggest that PRD may not be successful when soil porosity favors lateral spread of irrigation water or an insufficient volume of irrigation is applied at the time of the switch for restoration of the wet side to field capacity. In fact, when soil water status of the wet part of the root system is low, there is insufficient soil water in the dry part of the root system to maximize ABA export from the entire root system (Dodd et al. 2008 a,b). There is also some evidence that in low vigour vineyards PRD is unable to induce better agronomical output than the conventional deficit irrigation strategy, since the growth inhibition more pronounced in PRD than in DI will decrease source (leaves) to sink ratio below the optimum, resulting in yield losses without any improvement in berry quality (Lopes et al. own results). Moreover, Sadras (2009) in a meta-analysis of a broad range of horticultural crops showed that in general there was no improvement in the irrigation water productivity (yield per unit irrigation water applied) under PRD, as compared to DI.

3. PHYSIOLOGICAL RESPONSE TO MODERATE WATER DEFICITS IN GRAPEVINE

Under mild to moderate water deficits stomata closure is among the early plant responses, restricting water loss and carbon assimilation (Chaves et al. 2003). Direct effects on photosynthetic metabolism (Lawlor and Tezara 2009) and on the expression of a multitude of genes (Chaves et al. 2009) may also be present very early on. Under long-standing water deficits acclimation responses do occur, including those related to growth inhibition and to osmoregulation; these are key elements for the maintenance of plant water status and therefore plant carbon assimilation under water scarcity.

In grapevine, it has been reported for several varieties and different experimental conditions (greenhouse and field; short and long-term) that photosynthesis is quite resistant to water stress (Souza et al. 2003, 2005a; Chaves et al. 2007; Flexas et al. 2002). Under low-to-moderate water availabilities occurring under deficit irrigation, the maintenance of the activity of Calvin Cycle enzymes and of the Vcmax and Jmax values was generally observed (Souza et al. 2005a). However, when stress is intensified a decline in those parameters occurs, more markedly in Jmax (Maroco et al. 2002; Souza et al. 2005a), which can be a result of a decreased ATP production. Lawlor and Tezara (2009) raised the hypothesis that ROS produced under conditions of low CO2 and excess light might induce oxidative damage to chloroplastic ATPase.

Grapevine is prone to down-regulation of photosynthesis in the afternoon, phenomenon that might also occur in well-watered vines mainly as a result of stomatal closure in response to high vapour pressure deficit (VPD) and high irradiance (Correia et al. 1995) and/or to decreased stem hydraulic conductance (Salleo and Lo Gullo 1989; Vandeleur et al. 2009). Although several lines of evidence suggest that grapevines are resistant to photoinhibition (Correia et al. 1990; Chaumont et al. 1997; Souza et al. 2003; Flexas et al. 2001; Medrano et al. 2002), maximum efficiency of photosystem II (measured by the dark-adapted Fv/Fm) was shown to decline under intense drought (Quick et al. 1992).

Photosynthetic rates generally decline at lower pre-dawn water potentials than stomatal conductance, when grapevines are subjected to moderate water deficits. As a consequence, intrinsic water use efficiency (A/g_s or WUEi) is usually higher in vines under deficit irrigation (mild to moderate water deficits) than under well-watered conditions. This is reflected in a lower water use (WU) and higher WUE by the crop, an important aim of deficit irrigation strategies in vineyards (Gaudillère et al. 2002; Chaves et al. 2004; Souza et al. 2005b).

When water supply declines, stomatal guard cells respond to leaf water potential and both respond to and control the supply and loss of water by the leaves (Leuning et al. 2003). Under these circumstances, intercellular CO2 partial pressure (pi) can control stomatal opening via the supply of CO2 to the chloroplast or via the demand for CO2 by

photosynthesis. We usually observe that the decrease in g_s in response to mild water stress leads to a linear decline in transpiration (under constant VPD) and of pi, because CO2 demand by the chloroplasts (photosynthetic capacity) remains the same (Chaves et al. 2004). Under low light intensity but high air humidity, as it occurs in mornings or evenings, grapevine stomata may be widely open at low photosynthetic rates, leading to low intrinsic water use efficiency (WUEi). On the other hand, stomatal closure at midday, an important adaptation to high VPD in some species of xeric habitats (Maroco et al. 1997), may lead to an increase in WUEi when photosynthesis is maintained. This has been observed in grapevine (Souza et al 2003). When analysing WUEi it is therefore important to study it along the day. Field studies using var. Moscatel, Castelão and Aragonez (syn. Tempranillo) showed that deficit irrigation strategies (e.g. PRD and the conventional DI, both at 50% ETc) promoted an increase in WUE, when compared with fully irrigated grapevines (100% ETc), both in short-term (as expressed by the A/g_s ratio) and long-term (estimated via δ13C) (Souza et al. 2005b). An increase in WUE and related water savings under deficit irrigation was also reported in studies carried out in different grapevine varieties and in different locations (Dry et al. 2000; Stoll et al. 2000; Loveys et al. 2004; Poni et al. 2007; Marsal et al. 2008).

4. GENOTYPIC DEPENDENT RESPONSES TO WATER DEFICITS IN VITIS VINIFERA

It is acknowledged that the timing and intensity of the response to soil and atmospheric water deficits, namely in what concerns stomatal control, depends greatly on the genotype. This has profound implications in irrigation management, in particular the timing and amount of irrigation to optimize source-sink relationships, in order to achieve optimal fruit quality in each variety (Medrano et al. 2003; Poni et al. 2007; Chaves et al. 2007). *Vitis vinifera* L. is characterized by large genetic variability with several thousands of varieties/varieties being cultivated worldwide (Alleweldt et al. 1990; Gallet 2000; Shultz 2003). European countries like France, Spain or Portugal host a large number of native *V. vinifera* varieties. However, most of those genotypes remain uncharacterized,

which limits their use for breeding, for example to increase WUE or improve berry quality traits.

Genotype related differences in WUE and water stress resistance may arise from constitutive differences in leaf gas-exchange, plant capacity to osmoregulate and plant hydraulics. Photosynthesis, stomatal conductance and WUE_i were shown to vary with grapevine variety (Chaves et al. 1987; Shultz 1996, 2003; Bota et al. 2001; Soar et al. 2006; Palliotti et al. 2009). Still, variation in photosynthetic efficiency seems to be small (Bota *et al.* 2001), suggesting that genotypic variation in WUE is largely linked to diversity in stomatal conductance, both under well-watered and water deficit conditions (Gaudillère et al. 2002; Escalona et al. 1999; Chaves and Oliveira, 2004). Under drought conditions, a close relationship was found between stomatal function and plant hydraulics (Sperry 1986; Cochard et al. 2002; Sperry et al. 2002). Stomata keep water flow within safe limits preventing the plants to exceed those limits at any particular water potential, therefore avoiding xylem embolism (Sperry et al. 2002). Higher stomata sensitivity to water deficits may compensate for higher vulnerability to cavitation under drought (Schultz, 2003). *Vitis vinifera* shows high hydraulic conductivity in the main stem axis (Lovisolo et al. 2007). However, leaf hydraulic conductance can substantially constrain water transport, being a more important hydraulic bottleneck than the stem (Sack et al. 1993). It is also known that hydraulic conductance of roots and shoots influences stomatal regulation and plant transpiration (Aasamaa et al. 2001; Lovisolo and Schubert 1998; Rogiers et al. 2009). The distribution of vessel sizes varies with varieties and the larger sizes often results in higher sensitiveness to embolism under drought conditions (Chouzouri and Shultz 2005).

Leaf morpho-anatomy and related biochemistry (epicuticular wax composition, lipid composition, mesophyll thickness, etc) may also play a role in explaining plant adaptation to water stress (Syvertsen et al. 1995; Boyer et al. 1997; Cameron et al. 2006). Differences among *V. vinifera* have been reported in these characteristics (Schultz 1996; Moutinho-Pereira et al. 2007).

Grapevine is generally considered a “drought avoiding” species, with an efficient stomatal control over transpiration (Chaves et al. 1987; Shultz 2003). However, some genotypes have shown a better control of stomata than others in response to water deficits and accordingly have been classified as isohydric (drought avoiders or “pessimistic”); the others, showing lower control over stomatal aperture under water stress, were considered anisohydric, with an “optimistic” response (Schultz 2003; Soar et al. 2006). Schultz (2003) considered Grenache to be a nearly isohydric genotype showing a marked regulation of stomatal conductance to decreasing soil water, whereas Syrah exhibited a response closer to an anisohydric type. The same contrasting behaviour between Grenache and Syrah in response to atmospheric moisture stress was found by Soar et al. (2006), who attributed the higher sensitivity of stomata in Grenache to the higher concentration of ABA in the xylem sap as compared with Syrah. He provided evidence of a midday increment of the expression of key genes involved in the ABA biosynthetic pathway, significantly higher in the leaves of Grenache than in Syrah. This was not observed in the roots.

However, contradictory reports appeared in the literature showing that the same variety could behave differently depending on experimental conditions (see Table 1; and the review by Lovisolo et al. 2010). For example, var. Syrah and Grenache that exhibited an anisohydric and near-isohydric behaviour, respectively, in field experiments (Shultz 2003; Soar et al. 2006), did not display the same stomatal behaviour when experiments were performed with potted plants (Chouzouri and Shultz 2005).

Recent studies performed in our group also evidenced differences between varieties (Touriga Nacional, Trincadeira, Aragonez (syn. Tempranillo), Cabernet Sauvignon and Syrah, see Table 2) in the response of leaf stomatal conductance to deficit irrigation under field conditions. Stomatal conductance of Touriga Nacional remained the highest along the day (morning and afternoon) for similar leaf water potential, suggesting an anisohydric type of response (Fig. 1)**.** In contrast, Syrah showed the lowest conductance of the five varieties, particularly at noon, therefore exhibiting a near-isohydric response, contrary to earlier reports (Schultz 2003, Soar et al. 2006).

Table 1 List of grapevine varieties categorized as function of the response of the water potential to water deficit (iso or anisohydric), cultivated in soil (F) or in pots (P), with the correspondent range of values of water potential measured in each experiment.

Variety	Category	Set-up	Range of ψ (MPa)	References
Chardonnay	Anisohydric	F and P	-0.4 to -1.0	Tyerman (2007); Vandeleur et al. (2009); Rogiers et al. (2009)
Cabernet Sauvignon	Anisohydric	F	-0.7 to - 1.5	Williams and Baeza (2007)
	Isohydric	F	-0.25 to -1.5	Chalmers (2007)
Falanghina	Near-isohydric	F	-0.7 to -1.8	Giorio et al. (2007)
Kékfrancos	Near-isohydric	F	-0.1 to -1.2	Zsófi et al. (2008 ; 2009)
Grenache	Near-isohydric	F and P	-0.2 to -1.4	Shultz (2003); Santesteban et al. (2009)
	(Not clear)	P	-0.2 to -0.4	Chouzouri and Shultz (2005)
Lambrusco	Isohydric	P	-0.6 to -1.2	Poni et al. (2009)
Montepulciano	Anisohydric	F	-	Silvestroni et al. (2005)
Manto Negro	Isohydric	F	-0.05 to -0.7	Medrano et al. (2003)
	Anisohydric	-	-	Lovisolo et al. (2009)
Merlot	Anisohydric	F	-0.8 to -1.3	Williams and Baeza (2007); Shellie and Glenn (2008)
Portugiesier	Near-isohydric	-	-0.1 to - 0.9	Zsófi et al. (2008)
Riesling	Anysohydric	-	-	Lovisolo et al. (2009)
Sangiovese	Isohydric	F and P	-0.2 to -1.3	Poni et al. (2007); Silvestroni et al. (2005)
	Anysohidric	P	-0.55 to -1.3	Poni et al. (2007)
Seedless Thomson	Anysohidric	F	-0.7 to -1.3	Williams and Baeza (2007)
Semillon	Anisohydric	F and P	-0.4 to -1.8	Rogiers et al. (2009)

Soultanina	Isohydric	P	-0.15 to -0.8	Paranychianakis et al. (2004)
Syrah	Anisohydric	F and P	-0.2 to -0.8 -0.2 to -1.4	Shultz (2003); Chalmers (2007); Santesteban et al. (2009); Rogiers et al. (2009)
	(Not clear)	P	-0.2 to -0.4	Chouzouri and Shultz (2005)
Tempranillo (syn. Aragonez)	Isohydric	Fand P	-0.05 to -1.3	Antolin et al. (2006); Sousa et al. (2006); Medrano et al., 2003
	Near-isohydric	F	-0.2 to -1.5	Intrigliolo et al. (2005);
	Anisohydric	F and P	-	Santesteban et al. (2009); Lovisolo et al. (2009)
Touriga Nacional	Anisohydric	F	- 0.2 to - 1.5	Moutinho Pereira et al. (2004)
Viognier	Near-isohydric	F	-	Shellie and Glenn (2008)

Table 2 Pre-dawn leaf water potential (Ψ_{pd}, Mpa), leaf temperature (T_{leaf}, °C), leaf stomatal conductance to water vapour ($g_{sw,}$ $molH_2Om^{-2}s^{-1}$), net assimilation (An, $\mu molCO_2m^{-2}s^{-1}$), intrinsic water use efficiency (WUEi, $\mu mol\ CO_2.mol^{-1}H_2O$) and $\delta^{13}C$ ($^0/_{00}$) measured for five *Vitis vinifera* varieties. Aragonez (= Tempranilho) (ARA), Trincadeira (TRI), Syrah (SYR), Cabernet Sauvignon CAB) and Touriga Nacional (TOU). Vines were grown in field conditions in South Portugal (38º48'N, 7º29´W) and were 6-8 years old. Plants were grafted on the 1103-P rootstock, planted at a density of 4,000 plants/ha and trained on a bilateral Royal Cordon system. Leaf water potential was measured with a pressure chamber (Model 1000; PMS instrument Co., Corvallis, OR, USA) Leaf temperature was assessed by thermal imaging (IR Snapshot 525, 8-12 µm detector) at noon, and was immediately followed by measurements of leaf stomatal conductance using a portable photosynthesis system (Licor-6400, Li-COR Inc., USA) equipped with a transparent leaf chamber. Values of An and WUEi were determined at saturating light (1200 µmol m-2s-1), 360 ppm CO2 and block temperature of 25ºC, using a Licor-6400 equipped with a 6400-02B LED light source. Measurements were carried at beginning August 2007. Values are means ± STDEV (n=3-8 replicates) (Costa and Ortuño, unpublished).

Variety	Ψ_{pd}	T_{leaf}	g_{sw}	A_n	WUE_i	Delta ^{13}C
ARA	-0.25 ± 0.01	30.5 ± 0.2	0.076 ± 0.006	15.2 ± 0.8	59 ± 5	-27.60 ± 0.47
TRI	-0.10 ± 0.06	30.7 ± 1.6	0.074 ± 0.005	14.1 ± 0.5	54 ± 4	-27.96 ± 0.75
SYR	-0.19 ± 0.02	34.4 ± 1.5	0.049 ± 0.008	12.1 ± 0.7	93 ±12	-27.39 ± 0.66
CAB	-0.21 ± 0.07	31.4 ± 1.6	0.085 ± 0.008	12.4 ± 0.5	45 ± 4	-27.66 ± 1.07
TOU	-0.10 ± 0.02	29.5 ± 1.5	0.115 ± 0.007	15.6 ± 0.7	69 ± 14	-28.54 ± 0.69

For the variety Sangoviese, Poni and colleagues (2007) pose some questions regarding its classification with respect to response to water stress. The authors discuss in their

paper that since the first criteria to classify genotypes as being isohydric or anysohydric is how their leaf-water status (namely mid-day leaf-water potential) responds to a soil-water deficit treatment, they would classify the var. Sangoviese as anisohydric. However, several effects posed by partial rootzone drying on these vines, such as a fast cessation of shoot growth, leaves tending to assume a vertical orientation during midday to reduce light interception, and a pronounced and steady increase of WUE_i, have been reported as being more typical of an isohydric strategy.

Bearing in mind the available data, a classification of grapevine varieties as strict iso or anisohydric may prove inappropriate. It seems plausible that stomatal responses to water deficits in a specific variety will vary according to the particular combination of the rootstock, the climate (VPD and temperature) and the intensity and duration of water deficits. In fact, under prolonged water deficits more rigid cell walls may develop leading to a larger decline in plant water potential at midday, characteristic of the anysohydric response. Moreover, osmotic adjustment may contribute to the maintenance of open stomata at lower water potentials, by enabling an improved turgor in response to a slowly imposed water deficits. This combination of responses will interact with scion structural factors such as water-conducting capacity of stems and petioles to dictate response to water deficits.

This is an area of research deserving further investigation in order to clarify the relative importance of the factors involved in the dynamic response of stomata to water deficits.

5. LONG DISTANCE SIGNALLING OF WATER DEFICITS

Under soil drying plants reduce water use by stomatal closure and decreased growth. Hydraulic and chemical signals sent from drying roots to the shoot are involved in the regulation of these responses (Davies et al. 1994; Dodd et al. 1996; Liu et al. 2003). However, the relative importance of the two types of signalling in the control of stomatal aperture and leaf growth is still the subject of discussion. Depending on the species and/or experimental conditions hydraulic limitation may dominate over root

chemical signalling (Comstock 2002; Voisin et al. 2006; Neumann 2008; Ahmadi et al. 2009). This seems to be the case in some woody species, where chemical root-to-shoot signalling appears to be inefficient in controlling stomatal behaviour (Augé and Moore, 2002) or when other abiotic stresses co-occur with drought, as usually happens when plants are growing in their natural environment. Nevertheless, the primary role of a root-to-shoot hydraulic signal is generally followed by an increased ABA biosynthesis in the shoot that regulates stomata (Christmann et al. 2007) and leaf growth (Chazen et al. 1995; Neumann et al. 1997). Moreover, a great deal of evidence highlights the importance of ABA as root-sourced signal transported via the xylem and involved in stomatal regulation of droughted plants (reviews by Davies et al. 2005; Dodd et al. 1996, 2006; Wilkinson and Davies 2002). Even so, other compounds like the precursors of ABA (Sauter *et al.*, 2002; Lee *et al.*, 2006; Jiang and Hartung, 2008), low concentration of cytokinins (Shashidhar et al. 1996; Stoll et al. 2000; Hansen and Dorffling 2003), and changes in mineral composition or pH of the xylem (Wilkinson and Davies 1997; Hartung et al. 1998; Prokic et al. 2006; Jia and Davies 2007) might also be implicated in the regulation of water use at the leaf level (recently reviewed by Schachtmann and Goodger 2008). Much evidence suggests that xylem sap pH can indeed modulate stomatal and growth responses to root chemical signals produced in drying soils (Wilkinson and Davies, 1997*,* 2002; Wilkinson 2004). For acidic xylem sap pH, ABAH is taken by the leaf and metabolised or partitioned into alkaline compartments in the symplast of leaf cells, away from the sites of action of the hormone on stomata. Conversely, as pH increases, the proportion of ionised ABA transported in the xylem sap rises (not taken up by mesophyll cells) and so is maintained longer in the leaf apoplast adjacent to the guard cells, having a higher control on stomatal behaviour (Hartung et al. 1998; Wilkinson 2004). This effect is particularly important in grapevines since usually they have pH values close to the pKa of ABA (pH 4.8), as shown by Stoll et al. (2000) and Rodrigues et al. (2008). Indeed, work done with the grapevine variety Castelão (Rodrigues et al. 2008) provided evidence for a synergistic effect of increased pH and ABA explaining stomatal closure at berry maturity, whereas earlier in the season

(véraison) a low xylem pH was measured and no correlation between ABA and g_s was found (Fig. 2). In a recent work, Sharp and Davies (2009) found out that drought induced change in pH is more common in herbaceous than in woody perennials species. In fact, those authors studying 22 woody species only observed an increase in pH in 4 of them, the majority maintaining a pH similar to the well watered plants.

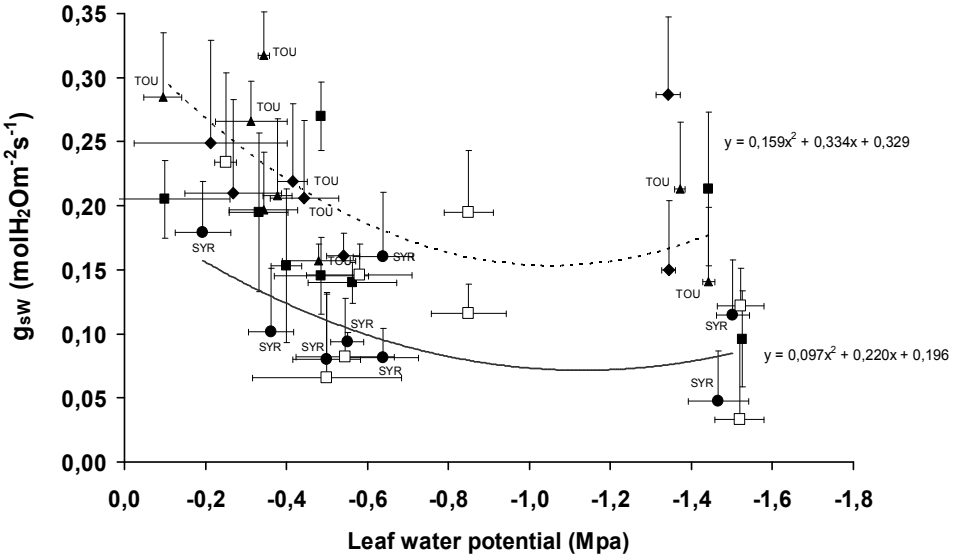

Figure 1 Relation between leaf stomatal conductance to water vapour (g_{sw},) and leaf water potential (Ψ) measured along the day (predawn and midday) for five different *Vitis vinifera* varieties, Touriga Nacional (▲), Syrah (●), Aragonez (Tempranilho) (□),Cabernet Sauvignon (♦) and Trincadeira (■).Vines were grown in field conditions in South Portugal (38º48'N, 7º29´W) and were 6-8 years old. Vines were grafted on the 1103-P rootstock, planted at a density of 4,000 plants/ha and trained on a bilateral Royal Cordon system. Measurements took place during the summer season (beginning of August) of three consecutive years: 2006, 2007 and 2008. Ψ was measured with a pressure chamber (Model 1000; PMS instrument Co., Corvallis, OR, USA) and g_{sw} was measured with a portable photosynthesis system (Licor-6400, LI-COR Inc., USA) equipped with a transparent leaf chamber. Horizontal and vertical bars indicate the standard deviation (n = 8). Lines represent regression lines estimated for the varieties Touriga and Syrah, as indicated.

Grapevine stomata also strongly respond to plant water status, through hydraulic tensions developed in the xylem affecting leaf turgor. Positive correlations between predawn water potential and maximum g_s have generally been found in grapevines

subjected to water deficits (Correia et al. 1995; Flexas et al. 1998; Rodrigues et al. 2008). Like in other species a decrease of shoot hydraulic conductivity has been shown to occur in water stressed grapevines (Schultz and Matthews 1988; Lovisolo and Schubert 1998; Lovisolo et al. 2002) and is linearly correlated with g_s under mild stress levels (Lovisolo and Schubert 1998). Moreover, it was shown that a decline in leaf water potential might enhance stomatal sensitivity to ABA. This interactive effect can explain the decrease in g_s observed at midday in grapevines growing under field conditions, including well watered ones, in spite of constant diurnal [ABA] in the xylem stream (Correia et al. 1995; Rodrigues et al. 2008).

When considering deficit irrigation, there is no clear picture of the relative importance of hydraulic and chemical signalling on plant response to water deficit. There are studies indicating a marked decrease of g_s in PRD grapevines relative to conventionally-irrigated vines, in spite of comparable shoot water status (Dry and Loveys 1999; Du et al. 2006), therefore suggesting the involvement of a non-hydraulic signal in stomatal regulation. Several other studies, however, did not find evidence for a more marked stomatal closure in PRD than in DI grapevines (Dorji et al. 2005; Souza et al. 2003; De la Hera et al. 2007; Marsal et al. 2008; Rodrigues et al. 2008). The higher water status of PRD plants may be derived from the observed restriction in vegetative growth of PRD plants (Santos et al. 2003 2005; Chaves et al. 2007), leading to lower plant water use and thus more water available in the soil near the root system. Differences in root architecture, with an increased ability to exploit deeper soil layers (Dry et al. 2000; Mingo et al. 2004; Santos et al. 2007), has also been reported as well as an increase of root hydraulic conductivity after root rewatering. In fact, recent work in an irrigated pear orchard showed that root sap flow on the wet-side of PRD plants was enhanced compared to control plants equally watered on both sides of the root system (Kang et al. 2003). Also, Green et al. (1997) observed in mature apple trees that previously dehydrated roots responded to irrigation by exhibiting higher sap flow rates than usually occurs when the entire root zone is watered. This increase in root hydraulic conductivity seems to be mediated by aquaporin activity (Martre et al. 2002; Lovisolo and Schubert 2006) since a significant

part of the radial water transport takes place through the cell-to-cell pathway (Martre et al. 2002; Siefritz et al. 2002).

Considering the causes for the observed restriction of vegetative growth under similar or better water status in PRD grapevines as compared to DI, chemical signals are the likely candidates to explain these results (Chaves et al. 2007). Such chemical root-to-shoot signalling probably involves a reduction of cytokinins (Kudoyarova et al., 2007) or an increase of ethylene (Sobeih et al. 2004). Cytokinins (CKs) are synthesized mainly in the roots (Aloni et al. 2005) and were shown to play an important role as long-distance signalling molecules (Schmulling 2002; Werner et al. 2003; Hirose et al. 2008). Dry et al. (2001) observed shoot growth inhibition in PRD grapevines in parallel with a marked decrease in the concentration of CK in shoots and roots. This effect was reversed by exogenous application of a synthetic CK. Similarly, a marked reduction in zeatin and zeatin riboside concentrations in roots, shoot tips and buds was found in PRD grapevines (Stoll et al., 2000) Although most results in other species also point to a decrease in the delivery of CKs to the xylem sap in water stressed plants (Shashidhar et al. 1996; Bano et al. 2003; Hansen and Dorffling 2003), there are exceptions with the opposite effect (a CK rise) (Pospísilová et al. 2005).

6. CHANGES IN BERRY GROWTH, METABOLISM AND COMPOSITION UNDER WATER DEFICITS

Water deficits influence berry development, metabolism and final composition, and its timing and intensity dictate the extent of alterations occurring in wine colour and flavour. Interestingly, water deficit was also shown to enhance photoprotection mechanisms in berries (Deluc et al. 2009). In general, mild water deficits were shown to have a positive impact on wine quality in red varieties (Bravdo et al. 1985). Under this context, deficit irrigation can provide the means to manipulate wine sensory characteristics. However, the effects of deficit irrigation on berry and wine quality will depend on the climatic characteristics during the growing season, the soil type, the grapevine variety and the timing of application (Dry and Loveys 1998; Santos et al. 2003, 2005).

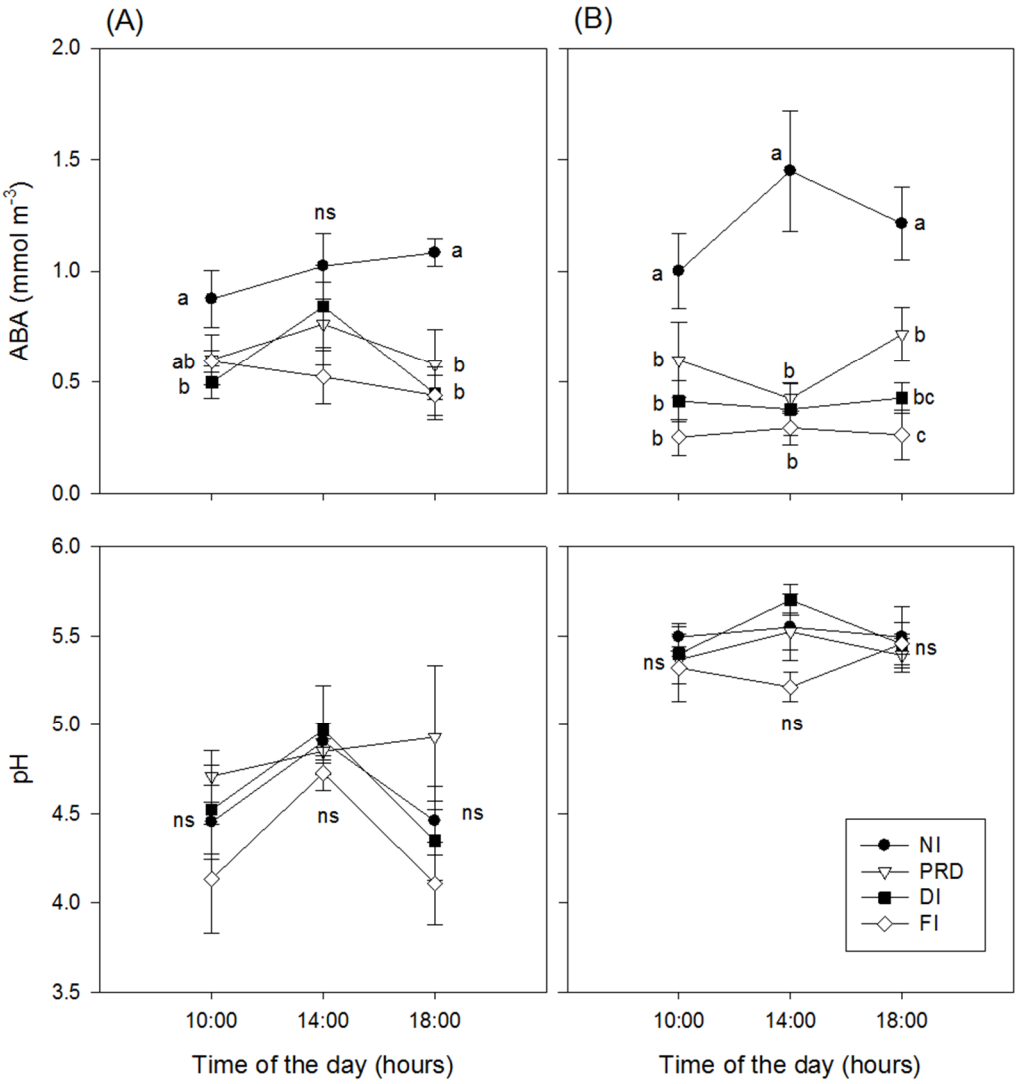

Figure 2 Diurnal changes in concentration of abscisic acid (ABA) in the xylem sap and pH of the xylem sap of field-grown Castelão grapevines in Pegões, Portugal, under four water treatments (NI, non-irrigated; PRD, partial rootzone drying; DI, deficit irrigated; FI, full irrigated), measured on two days of the 2002 growing season, (**A**) veraison (25 July) and (**B**) mid-ripening (22 August). For each measurement time values are mean of four measurements. Error bars indicate the standard error. Different letters show statistically significant differences among treatments at $p < 0.05$. (Rodrigues et al. 2008).

Transcriptional analysis of grape berries from vines subjected to moderate water deficits at the end-ripening stage showed alterations on mRNA expression patterns particularly

associated with cell wall, sugar and hormone metabolism (Deluc et al. 2007). The most profound alterations were related to ethylene, auxin and abscisic acid, but an enhancement of the expression of several genes of the phenylpropanoid pathway was also observed.

The impact of water deficit on grape berry proteome was reported by Grimplet *et al.* (2009). These authors studied the alterations observed in the skin, pulp and seed proteomes of fully ripe berries when comparing water-deficit vines (no irrigation) with well-watered plants (irrigation from *pre-véraison* to the end of berry maturity) and showed that 7% of pericarp proteins were water-stress responsive. Using such an approach, we are currently studying the proteome dynamics of grapevines of the var. Aragonez (syn. *Tempranillo*) along berry development using three irrigation strategies. When comparing berries of full irrigated (FI) vines with the ones from deficit irrigated (RDI) and rainfed (NI) vines, several proteins were identified as stress responsive. One such protein was vacuolar invertase (GIN1), which was significantly down-regulated under NI and RDI when compared with FI conditions (Fig. 3). These alterations were observed at green stage (pre-*véraison*) and *véraison*. Moreover, the peak of expression of this protein that was reported to occur at *véraison* by others (Giribaldi et al. 2007; Deluc et al. 2007; Negri et al. 2008) was observed later in RDI than in FI berries. These results suggest that water availability modulates not only the amount, but also the timing of protein expression. It suggests as well that changes taking place very early on during berry development, such as at the green berry stage, may have a profound effect on the final berry maturity (Francisco *et al.*, 'unpubl. res.').

Vine water status is known to influence fruit composition through an indirect effect on berry size, and therefore the ratio of skin to pulp, which increases in the smaller berries of the vines subjected to water deficits (Bravdo et al. 1985, Kennedy et al. 2002). There is however a direct, possibly greater effect, on skin tannin and anthocyanin contents (Roby et al. 2004). Proteomic studies in berries from grapevines subjected to different irrigation treatments that suggest that metabolic differences in response to water status

occur at early stages of berry development (Francisco et al., 'unpubl. res.'), confirm that they are partly independent of the effect on berry size.

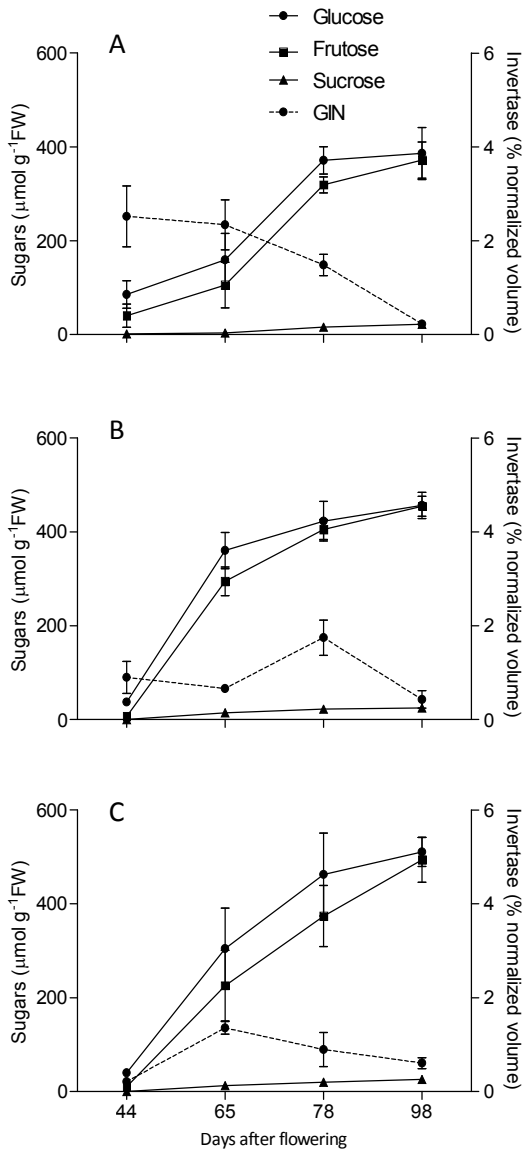

Figure 3 The influence of water deficit on sugar metabolism and vacuolar invertase (GIN1) expression profile along fruit ripening. (**A**) Plants under full irrigation conditions; (**B**) plants under regulated-deficit irrigation conditions and (**C**) plants under no irrigation but rain-fed. 2-DE spot volume is represented as % normalized volume. Symbols represent means ± SE (n =3).

Berry growth

Grape berry is a non-climacteric fruit with a double sigmoid growth curve (Coombe 1976). Stages I and III of growth are separated by a lag phase (stage II). During stage I, imported carbohydrates are used for seed development, cell proliferation and expansion, and synthesis of organic acids (Coombe 1992). At this stage the berry is exclusively connected to the vine through the xylem, and the impact of water deficit on berry growth is thought to occur directly by changes in water import by the xylem, which possibly induces a decrease in mesocarp cell turgor (Thomas et al. 2006). Consequently a reduction in the expansion of grape berries takes place. However, it is also possible that the ABA synthesized under water stress limits cell-division and consequently small berries are produced. The second hypothesis correlates well with the observed inhibition of grape development following water deficit at pre-*véraison*. This leads to a cascade of events culminating in earlier grape ripening (e.g. accelerating sugar and anthocyanin accumulation and malic acid breakdown) (Castellarin et al. 2007a, b). The beginning of the second phase of berry growth (stage III), known as *véraison*, is characterized by softening and colouring of the berry and size increment. After *véraison* reduction in berry size due to water deficits is probably the result of more than one mechanism (Thomas et al. 2006). At this stage, the berry's connectivity to the vine is via the phloem (Thomas et al. 2006). Moreover, reduction of berry size might be only indirectly caused by water stress, through a decrease in photosynthesis (Wang *et al.*, 2003). Post-*véraison* water deficit increases the proportion of whole-berry fresh mass represented by seeds and skin (Roby and Mathews 2004) and berries present 'thicker skins' at harvest probably due to a decrease in the activity of pectin methylesterase enzyme (Deytieux-Belleau et al. 2008), as was shown in water stressed tomato cherry fruit (Barbagallo et al. 2008). This results in higher content of skin-based constituents (e.g. tannins and anthocyanins) on a berry mass basis and as a consequence, the must from those berries is much richer in skin-derived extractives (Chatelet et al. 2008).

Sugar and organic acids accumulation

Grape quality largely depends on sugar/acid balance at harvest. Prior to *véraison*, most sucrose (Suc) imported into the berries is metabolized with little if any storage. However, following *véraison* hexoses accumulate in the berries at high concentration (1M or even more). Grapevine is thought to be a symplastic phloem 'loader' due to the presence of plasmodesmata connecting mesophyll cells with phloem associated cells (Gamalei 1989). It has been suggested that the symplastic connections via plasmodesmata between the sieve tubes and the mesocarp cells remain for quite a long period during berry development. Phloem unloading seems to occur via efflux into the apoplast and subsequent uptake by sink cells. Suc from the phloem can be imported from the apoplast via direct Suc transporters or it can be hydrolysed to Glc and Fru by cell wall bound invertases and taken up by monosaccharide transporters. In grape berry, it is known that invertase expression considerably precedes the onset of sugar accumulation (Davies and Robinson, 1996). This suggests that the triggering of ripening depends on the activation of sugar transporters (for a review, see Conde et al. 2007).

Moderate water deficit promotes sugar accumulation either as a result of inhibiting lateral shoot growth, which induces a reallocation of carbohydrates to fruits, or as a direct effect of ABA signalling on fruit ripening (Coombe 1989). Indeed, experimental evidence suggested activation of ABA-mediated uptake of hexose (Deluc et al. 2009). However, up to now the mechanisms underlying hexoses accumulation under water deficit have not been totally elucidated.

The effects of water deficit on sugar content of grapevine berries are variety dependent (Gaudillère et al. 2002). For example, no significant changes were observed in Merlot sugar content under water deficits, while a significant increase in sugar content was observed in Cabernet Sauvignon berries (Castellarin et al. 2007a, b). Similarly, Deluc et al. (2009) observed an increase in berry sugar content under water deficits in Cabernet Sauvignon but not in Chardonnay. This may be explained either by differences in vigour, and therefore source/sink equilibrium, between varieties, or by different mechanisms underlying the response of grape berry development to water limitation according to

the timing and intensity of water stress imposition. Indeed, it was shown that water deficit has more effect on berry sugar accumulation when imposed before *véraison* (Keller 2005; Keller et al. 2006).

In most cases, no titratable acidity changes have been observed in the must from moderately water stressed vines (Mathews and Anderson, 1989; Esteban *et al.*, 1999). However, some studies report a reduction of titratable acidity due to deficit irrigation as compared with full irrigation (Sheltie 2006; Santos et al. 2007). Malate/tartarate ratio is in general lower due to malate breakdown in vines with low water status (Mathews and Anderson 1989).

Polyphenols

Among the different classes of polyphenols present in grape berries the most important are flavonoids (anthocyanins, flavonols and proanthocyanidins also called condensed tannins) and stilbenes. They are mainly localized in exocarp and seed endocarp tissues and it is well known that vine water status affects accumulation of polyphenols in these tissues. Regulating grapevine water deficit is a powerful tool to manage the amount of these compounds and improve wine quality (Kennedy et al. 2002).

Anthocyanins are synthesized via the flavonoid pathway in the berry skin of red grapevines from the onset of ripening (*véraison*) and are non-existent in white grapevine varieties due to a multiallelic mutation (Walker et al. 2007). Water deficits has been considered as an enhancer of accumulation of anthocyanins, through the stimulation of anthocyanin hydroxylation, probably by up-regulating the gene encoding the enzyme F3'5'H (Mattivi et al. 2006; Castellarin et al. 2007b). This enzyme converts hydroxylated anthocyanins (cyanidin and delphinidin) into their methoxylated derivates (peonidin, petunidin and malvidin) (Kennedy et al. 2002; Castellarin et al. 2007b). Indeed, the major anthocyanins synthesised in the berries under water deficits are peonidin 3-O-β-glucoside and malvidin 3-O-β-glucoside, because methoxylation of delphinidin to produce its derivate petunidin rarely occurs (Castellarin et al. 2007b; Deluc et al. 2009).

Water stress seems to have a larger impact on anthocyanin composition than on its total concentration. Early imposition of water stress led to increased sugar accumulation,

which accelerates anthocyanin synthesis (Castellarin et al. 2007b), probably due to 'sucrose boxes' in the promoters of *LDOX* and *DFR* genes (Gollop et al. 2001; 2002). Gene regulation of the anthocyanin pathway was known to be affected by the timing of imposition of water deficit (Castellarin et al. 2007a).

Flavonols play a fundamental role on grape quality, as they act as co-pigments with anthocyanins and stabilise colour in young red wines (Boulton 2001). Flavonol biosynthesis is closely related to that of anthocyanins (Jeong et al. 2006). However, in contrast with anthocyanins, a low number of flavonols was identified and available data is limited to a few grape varieties (Mattivi et al. 2006). The main flavonols reported in grape berries are quercetin-3-glucoside and quercetin-3-O-glucuronide (Downey et al. 2003). Deficit irrigation was reported to affect moderately flavonol synthesis in red grapevines (Grimplet et al. 2007). In turn, the timing of water deficit does not change flavonols content (Kennedy et al. 2002). Recently, Mattivi et al. (2006) suggested that anthocyanins and flavonols share the same biosynthetic enzymes. This may indicate that, like anthocyanins, changes under water deficits may occur rather in the flavonol composition than its accumulation. More recently, in a white grapevine (var. Chardonnay), flavonol concentrations were reported to increase under water deficits, which was not the case in a red grapevine (var. Cabernet Sauvignon) in the same study (Deluc et al. 2009). This suggests a greater need for berry photoprotection in these varieties, as previously shown in apples with low levels of anthocyanins (Merzlyak et al. 2008).

Proanthocyanidins or condensed tannins (PAs) are flavan-3-ol oligomers. They are important sensory components, providing wine with bitterness and astringency. However, little is known about PAs (review Dixon et al. 2005; Xie and Dixon 2005) and a standardized measure of tannins has not yet been adopted (Downey et al. 2006). Besides, changes occurring in proanthocyanidins during development of the grape are complex, involving increases in the degree of polymerization, in the proportion of (–)-epigallocatechin extension units, and in polymer-associated anthocyanins (Kennedy *et al.*, 2002). PAs appear to be only slightly affected by water deficit (Downey et al. 2006)

and the increases in skin tannin that accompany water deficits appear to result more from differential growth sensitivity of the inner mesocarp and the exocarp than from direct effects on phenolic biosynthesis (Roby et al. 2004). The concentration of seed tannins on wine is not known (Matthews and Nuzzo 2007). Moreover, few works reported whether or not water status influences seed proanthocyanidin content. Two studies performed with the same variety (although in different environments) did not show any significant effects of water deficit on seed proanthocyanidins (Kennedy et al. 2000; Geny et al. 2003). A gene expression study undertaken by our team (Zarrouk et al. 'unpubl. res.'), demonstrated differential expression during grape berry development of the *ANR* gene in grape seeds and a slight down-regulation under water stress.

Stilbenes belong to the non-flavonoid class of phenolic compounds. Generally, stilbenes are considered as phytoalexins, and their formation in grape leaves was correlated with disease resistance. Resveratrol is considered the most bioactive compound within the stilbenes group in grapevines (Bavaresco et al. 2008). In grape berries, resveratrol synthesis is catalyzed by stilbene synthase (STS), which shares the same substrates used by chalcone synthase for flavonoids production (Versari et al. 2001). It is accumulated mainly in the grape skin and seeds, and it has been found both in red and white grapes on a large range of concentrations, depending on biotic and abiotic conditions (Jimenez *et al.*, 2007). Some conflicting results arise on the effects of water deficit on resveratrol synthesis. Research conducted by Vezzuli and co-authors (2007) observed little effect of drought on resveratrol concentrations in grape berry skin. An increase in mRNA abundance of *STS* was reported by Grimplet et al. (2007), which suggest an increase in resveratrol accumulation (Versari et al. 2001). Under moderate water deficit, gene expression of *STS1* and *STS2* in grape seeds showed an up-regulation at berry maturity (Zarrouk et al. 'unpubl. res.').

Aromas

The aroma that builds-up in grapes results from several compounds (terpenoids and their derivatives, esthers, aldehydes and thiols) stored as non-volatiles precursors mainly in exocarp vacuoles.

The influence of the irrigation strategy on grape berry aromas has not been the subject of much research. However, two major studies suggest that deficit irrigation alters several sensory attributes of the wine as well as the concentration of carotenoids and their derivatives in berries, as compared to standard irrigation grapevines (Chapman et al. 2005; Bindon et al. 2007). Chapman et al. (2005) reported that water deficits led to wine with more fruity and less vegetal aromas than those from vines with high water status,, in the variety Cabernet Sauvignon. According to these authors water deficits may have led to a greater flux of carbon through alternate biosynthetic pathways leading to an increase in amino acids (precursors of esters in wines) and in carotenoids, resulting in more fruity aroma. Bindon et al. (2007) observed that deficit irrigation led to an increase in the concentration of hydrolytically released C_{13}-norisoprenoids (β-damascenone, β-ionone and 1,1,6- trimethyl-1,2-dihydronaphthalene) in Cabernet Sauvignon grape berries at harvest. Furthermore, transcriptomic analysis of genes encoding enzymes involved in the biosynthesis of volatile compounds revealed an increase in the transcript abundance of one terpenoid synthase, one carotenoid cleavage dioxygenase and several lipoxygenases under conditions of water deficits (Deluc et al. 2009). However, we should emphasize that the correlation of enzyme transcript abundance with the reaction products they catalyse is not straightforward, given the complexity of gene regulation, enzyme activity modulation and differential expression of multigenic families.

7. CONCLUSIONS AND WAY FORWARD

Deficit irrigation is an efficient strategy to improve WUE and control vigour in grapevine, allowing an optimal grape maturity and therefore a high wine quality. It is now acknowledged that the efficiency of deficit irrigation (whatever the sub-type) in modulating WUE, growth and grape berry composition is dependent on the variety characteristics (namely its vigour and drought avoiding traits), the type of soil and the prevailing weather (rainfall and temperature). More in-depth and wider studies of varieties in response to environmental stresses are instrumental to the understanding of

grapevine adaptation to more arid climates. Further knowledge on berry development, including the timing for the accumulation of various berry components, and their dependence on water availability, is critical for an optimal choice of irrigation strategy. Proteomic and transcriptomic studies are providing new avenues for that understanding. The data already available suggest that water deficits interact with development to alter the expression of genes responsible for some grape berry compounds and metabolite transporters. Although some of those changes seem to be transient it is plausible that they will have an impact on berry maturity and the final wine quality.

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Chapter VI

Final discussion

This section identifies the major contributions described in the dissertation and discusses future perspectives.

The study of the variations in gene and protein expression during grape maturation is not only relevant from a basic biological perspective but also from an applied point of view. Indeed, only when we fully apprehend the molecular events of ripening we can aim to successfully manipulate it. The recent transcriptome and proteome-wide studies led to new insights on the ripening events with the induction of genes/proteins related to several pathways, such as those involved in the regulation and biosynthesis of secondary metabolites, sugars transport and cell wall metabolism (reviewed by Conde et al. 2007; Tornielli et al. *in press*). In the light of the available data, grape berry ripening is a coordinated and compartmentalized process that involves berry growth, hexoses and anthocyanins accumulation (in red cultivars), berry softening, organic acid metabolization, apart from other metabolic events leading to accumulation of amino acids, flavour and aroma compounds (Kanellis and Roubelakis-Angelakis 1993). Still, the genetic control of ripening is not fully understood, although much of the effort made by the grape scientific community has been directed to characterize which is/are the molecular switch(s) of the ripening events. This idea of a coordinated process, where anthocyanins start to accumulate at the same time as sugars accumulate in berry flesh has been recently challenged (Castellarin et al. 2011). It was observed in Alicante Bouschet, a 'teinturier' variety that colour development starts in the berry flesh and then progresses into the skin. Future experiments will for sure clarify if this is an atypical behaviour of an atypical variety or if the ripening process in fact starts in the flesh tissues.

In the present study, the use of regulated-deficit irrigation (RDI) as compared with non-irrigation (NI) and fully irrigation (FI) was imposed as a way to manipulate the ripening process (Chapters II and III). Moreover, due to the relevance of anthocyanins metabolism as a quality trait in winemaking grapes we also pursued the challenge of identifying for the first time in *Vitis vinifera* an ABC transporter putatively involved in vacuolar sequestration of anthocyanins (Chapter IV). Our first objective was to evaluate

the impact of WD on grape berry quality parameters such as sugars, organic acids and anthocyanins (Chapter II and III). The physiological impact of the water deficit (mild to moderate) applied in the field to grapevines (RDI and NI) was not detrimental to parameters such as sugars and organic acids content. However, an early transition to ripening in RDI berries was observed since at 65DAF the accumulation of anthocyanins was detected and significantly different from berries exposed either to FI or NI conditions. Moreover, organic acids metabolization started earlier in WD berries as compared to FI vines.

The second objective of this thesis was to evaluate how water status conditions regulate, at the transcriptional level the major events that occur at grape berry exocarp during ripening and how this regulation may explain the observed alterations on grape quality traits such as anthocyanins and sugars.

At the transcriptional level we could not get a convincing explanation for the anticipation of anthocyanins accumulation in RDI berries as compared to NI berries. However, since the temporal lag between the first and the second sampling date (around 3 weeks) was considerable, we assume that some important trends in gene expression may have occurred and were not recorded by us. Still, one interesting observation was made regarding *MYBA3,* a non-functional VvMYB transcriptional factor (Walker et al. 2007). Despite the fact that *MYBA3* was highly expressed under both RDI and FI conditions throughout berry ripening, under NI a 2-fold induction was observed at 44 and 65DAF. This MYB was proposed to act during grape berry pigmentation as a competitor to the functional anthocyanins regulators *MYBA1* and *MYBA2* isogenes (Fournier-Level et al. 2009), although further experimental evidence is needed to support this hypothesis. Castellarin et al. (2011) emphasized that the role of *VvMYBA* in the regulation of anthocyanins biosynthesis may be oversimplified in grape berries, since in other model systems this biosynthetic pathway is under the cooperative regulation of WD40, bHLH and MYB transcription factors. Still, it is worth mentioning that at the transcriptional level the evidence points to flavonols induction under WD as compared

to FI conditions (Chapter II). This can be related to grapes exposure to sunlight (Fujita et al. 2006), since WD and particularly NI, modify vine canopy architecture favouring clusters exposition to solar radiation. So, although only a limited number of metabolites were determined in the scope of this thesis, there are many compounds related to berry quality which could have been influenced by WD.

Concerning hormonal regulation the observations regarding ABA metabolism at transcriptional level were particularly relevant, since they suggest that ABA may be internally regulated by a dynamic balance between biosynthesis and catabolism events during berry ripening. There is evidence that exogenous application of ABA can hasten the initiation of ripening (Matsushima et al. 1989; Wheeler et al. 2009). ABA is not only the major operating signal during drought stress, but in coordination with sugars may have a role in grape fruit development and ripening (Carrari et al. 2004; Gambetta et al. 2010). Transcripts associated with ethylene, auxins, brassinosteroids and cytokinins were also shown to be under WD regulation. The expression profile of transcripts involved, e.g., in signalling, transcriptional regulation (e.g. transcription factors), flavonoids, sugars or cell wall metabolisms showed that they undergo dynamic changes throughout the course of berry ripening. The fact that trehalose associated-transcripts were modulated by ripening and WD conditions suggests the participation of trehalose-6-phosphate (T6P) a 'sugar signal' (Paul 2007) in grape maturation processes. T6P has been described to participate in sugars utilization and starch metabolism and to interact with other signalling pathways, including those mediated by cytokinin and ABA (reviewed by Paul 2007). Finally, much of our findings at the transcriptional level provide clues for further functional analysis studies that could help to better understand the ripening events. The characterization of berry exocarp (skin) proteome and the identification of the major variations observed during grape ripening and under WD conditions were presented as the third major objective of this thesis (Chapter III). Proteomics-based technologies have been successfully applied to grapevine for the analysis of berry development (Sarry et al. 2004; Deytieux et al. 2007; Giribaldi et al. 2007; Negri et al. 2008; Zhang et al. 2008; Martínez-Esteso et al. 2011a,b). These studies

have improved our understanding on the dynamics of most relevant proteins in grape berries. However, WD effect on the ripening events was so far described in only the study carried out by Grimplet et al. (2007), where a survey of expression patterns of pericarp and seed tissue specific proteins was performed in fully mature berries. Moreover, we performed our studies in Aragonez (syn. Tempranillo), which is one of the most relevant varieties of the Iberia Peninsula. Varietal differences seem to be important, as shown by Sarry et al. (2004) who compared 6 grapevine varieties and concluded that 30% of the grape berry detected spots were not common to all genotypes.

From the 74 proteins identified in our study the major functional classes were represented by carbohydrate and stress-related proteins. The expression pattern of vacuolar invertase (GIN1) in grape berries (high at early stages of berry development) has been associated with the onset of ripening and with the shift of phloem unloading from symplastic to apoplastic (Zhang et al. 2006), with the concomitant increase of cell wall invertases activity (Davies and Robinson 1996). Our proteome data revealed that GIN1 was down-regulated after 65DAF under FI conditions, whereas under RDI and NI this trend was already observed at 44DAF. Interestingly, at transcriptional level the data do not corroborate this observation in what regards *GIN1* (VVTU13187_at) expression profile under WD conditions (Figure 9, Chapter II). Since the accumulation rate of hexoses is higher under RDI and NI between 44 and 65DAF, these results suggests that GIN1 may be under post-translational regulation (PTMs). In fact, several reports indicate that invertases may be developmentally regulated by proteolytic degradation or a specific mechanism via formation of invertases complexes with proteinaceous invertase inhibitors (Rausch and Greiner 2004; Jin et al. 2009). Invertase/pectin methylesterase inhibitors (PMEI) are members of a large family named PMEI-related proteins (PMEI-RPs; Hothorn et al. 2004) and our results indicate (Chapter II, Additional file 1) that they may be potentially involved in such regulation. However, their exact role during fruit ripening and more precisely as invertase inhibitors remains to be clarified. Interestingly, in

Arabidopsis, ABA was proven to regulate invertase activity by down-regulating its inhibitors (Koh et al. 2008).

Under RDI conditions a group of 8 proteins showed specific up-regulation at 65DAF. We propose that this group of proteins be assigned as *signature* proteins that characterize the onset of the ripening events. This group included among other proteins an alcohol dehydrogenase 1 (ADH1), a S-adenosylmethionine synthetase (SAM) and a thaumatin-like protein (TLP1). One of the questions that emerged was if the microarray data (Chapter II) could complement some of these observations. *ADH1* (VVTU17579_s_at) was in fact positively modulated during fruit ripening under both FI and RDI conditions. However, it was *ADH6* (VVTU11849_s_at) that showed a similar profile to the one described at the proteome level. Sarry et al. (2004) observed that ADH was one of the identified proteins that were differentially expressed in the six varieties studied, but it also showed how complex can be to precisely determine ADH isoforms in 2DE gels. ADH1 belongs to a multigenic family and in *V. vinifera ADH1-3* are the best characterised genes during berry development (Tesnière et al. 2000). *VvADH1* and *VvADH3* are described as being expressed up to véraison and down-regulated thereafter, whereas *VvADH2* is up-regulated during ripening (Tesnière et al. 2000), although a previous report from the same research team (Sarni-Manchado et al. 1997) showed in the same variety that *ADH1* was up-regulated at the inception of ripening. While further validations are required to confirm that the identified spot is in fact ADH1, the microarray data supports this observation, suggesting that different grapevine varieties may express different ADH isoforms throughout fruit ripening, what may have some biological meaning.

In what concerns *SAM* (VVTU18255_s_at) expression, an induction at 65DAF under both RDI and NI was observed. However, under NI conditions at the protein level this induction was not statistically significant. We also observed that *1-aminocyclopropane-1 carboxylic acid oxidase* (*ACO*; VVTU2507_s_at) was transiently up-regulated under RDI; altogether the transcriptional and protein evidence suggests that a transient peak of ethylene may have occurred under RDI conditions, favouring the emerging role of

ethylene during the ripening process (reviewed by Davies and Böttcher 2009). In the case of the thaumatin-like protein at the transcriptional level we observed that WD induced an up-regulation of *TLP*1 (VVTU40583_s_at), its expression being enhanced at 65DAF under both RDI and NI. However, the highest relative expression was observed under NI throughout berry ripening, what contrasts with the protein data. This may suggest that TLP1 is under PTMs control. Likewise, chitinase IV (which together with TLP1 are the most abundant proteins expressed in exocarp tissues) had at the transcriptional level, a 20-fold induction at 65DAF in all water status conditions. This was followed by a stable expression thereafter, corroborating previous results (Robinson et al. 1997). At the protein level TLP1 and IV chitinase were described as up-regulated from véraison onwards (Deytieux et al. 2007; Negri et al. 2008) and in both cases more than one isoform was detected, suggesting that in fact these proteins are under PTMs regulation. PTMs of proteins largely increase protein complexity and dynamics, resulting in the intricate regulation of biological events (Kwon et al. 2006).

The results provided in Chapter II and III offer a representative picture of the transcriptomic and proteomic dynamics of grape skin and an overview of the Aragonez grape ripening maturation biology. It was clearly showed that these two approaches provide complementary information that may be a valuable tool for future studies. The next challenge will be to elucidate the true biological meaning of all the data that were generated. It will then be possible to improve our understanding of the mechanisms that are operating during grape berry ripening and on the influence due to grapevine water status.

As already stated, anthocyanins constitute one of the most important secondary compounds present in grape berries. Although the mechanisms used by plants to transport these compounds into the vacuoles are still not clearly established, plant ABC transporters and GSTs have been described to participate in such process (reviewed by Grotewold and Davies 2008). Only recently, two grapevine Multidrug and Toxic Extrusion (MATE) proteins were shown *in vitro* to transport acylated (Gomez et al. 2009) but not the glucosylated anthocyanins forms which represent the majority of the

anthocyanins biosynthesized in grape berries. The data regarding an ABCC subfamily member of the ATP-binding Cassette superfamily from *Zea mays* (ZmMRP3) strongly suggested a role for ZmMRP3 in anthocyanin vacuolar sequestration although, no biochemical evidence had been presented (Goodman et al. 2004). This was our fourth and last objective; the initial study hypothesis was to find out if the closest *Vitis vinifera* homologue of ZmMRP3, VvABCC1 had the ability to transport the major anthocyanins forms (Chapter IV). We have confirmed this hypothesis, showing that VvABCC1 is able to transport *in vitro* glucosylated anthocyanins. Moreover, we also showed that this transport is ATP, GSH and time-dependent. The expression of *VvABCC1* was evaluated by qRT-PCR in both exocarp and mesocarp tissues. The expression of *VvABCC1* in mesocarp tissues, where anthocyanins do not accumulate, suggested that this protein functions beyond anthocyanin transport. Since IAA-Asp is an organic anion, hence a potential substrate for ABCC transporters, and since it is known that ABC proteins can transport many unrelated substrates (Rea 2007), it was further hypothesised whether VvABCC1 could also act as an IAA-Asp transporter, what was also experimentally confirmed (Chapter IV). This work is the first biochemical proof of the involvement of an ABC protein from the ABCC subfamily in the vacuolar sequestration of both anthocyanins and IAA-amino acid conjugates, supporting its involvement in the grape berry ripening process. One drawback of these results was that, until now, we could not confirm the vacuolar localization of VvABCC1 (work *in progress*). These results raise new questions that still need to be addressed with further research. Is there a specificity of VvABCCs in what regards the different glucosylated anthocyanins forms in grape berries? Which is the precise role of GST/GSH on the anthocyanins transport into the vacuole? Do environmental cues, such as WD, influence *VvABCCs* expression?

In conclusion, the use of post-genomic technologies enabled to unveil some of the main biochemical and molecular events of the complex grape berry ripening programme, that were altered by water status conditions. Still much work remains to be done until the true biological meaning of all the presented data are elucidated.

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ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

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